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Preface

This volume describes recent advances in the bioconversion of lignocellulosics. It starts with two articles on genetics and properties of cellulases and their reaction kinetics and mechanisms. The cost of cellulases has been a hindrance to large scale use of enzymatic hydrolysis. Two articles on cellulase production by submerged fermentation and by solid state fementation are included to describe the state of the art in this area. Dilute acid hydrolysis of cellulose continues to be of interest as well as potentially useful. The most recent advances in this area is also covered. A great deal of progress has been made in genetic engineering for improved regulation of xylose fermentation by yeasts. An article on genetically engineered *Saccharomyces* for simulteaneous fermentation of glucose and xylose describes the importance advances made in production of fuel ethanol from lignocellulosic biomass. In recent years, there has been increasing interests in recycling and the reuse of scrap paper as well as environment considerations. A contribution is presented which describes the research perspectives in that area. Finally, recent advances in the use of lignocellulosic biomass for the production of ethanol and organic acids are presented in two articles.

Renewable resources are inevitably of great importance in the years to come. There is a never-ending search for better living conditions for human beings. The more resource materials can be recycled, the richer we will be. Bioconversion of lignocellulosics, natural and man-made, is an important link in that cycle. Extensive use of renewable resources will also slow down continued deterioration of the environment.

Advances are being made as this volume is being put together. Another volume on the same subject, perhaps, should be prepared in another ten years or ever sooner.

March 1999 George T. Tsao

Contents

Genetics and Properties of Cellulases

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Cellulases are enzymes which degrade the insoluble, abundant polymer cellulose. In order to perform this task bacteria, fungi, plants and insects have developed a variety of different systems with multiple cellulases. In this review the similarities and differences of these enzymes are summarized based on the burgeoning information gained in recent years from amino acid sequences, three dimensional structures and biochemical experiments. The independent cellulases of aerobic organisms are contrasted with the cellulosomes of anaerobic organisms. The ability of different enzymes to synergize with each other is discussed along with the role of the different types of enzymes in cellulose degradation.

Keywords. Cellulosome, Endoglucanase, Cellobiohydrolase, Synergism, Mechanism, Regulation, Structure, Application

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List of Abbreviations

CBD cellulose-binding domain
CMC carboxymethyl cellulose
SC phosphoric acid swollen cellulose

1 Introduction

Cellulose is the most abundant polymer on earth with an estimated 10^{12} metric tons produced each year by plants [1] and cellulases produced by fungi and bacteria are responsible for most cellulose degradation [2]. Cellulose is a linear homopolymer of β1–4 linked glucose residues. There are stereochemical differences between adjacent glucose residues so that the repeating unit in cellulose is the disaccharide, cellobiose, and the main product of the enzymatic hydrolysis of cellulose is cellobiose. Cellulose is difficult to degrade because cellulose molecules can form tightly packed, extensively hydrogen-bonded regions called crystalline cellulose [3, 4]. The crystalline regions are believed to be separated by less ordered amorphous regions, but these still contain many hydrogen bonds. Cellulose is insoluble and oligomers containing more than six residues are also insoluble. The resistance of cellulose to degradation may be responsible for the large number and types of cellulases produced by cellulose degrading organisms. There are two basic types of cellulases, endo- and exocellulases. Endocellulases have a more open active site cleft and can bind at any available point along a cellulose molecule hydrolyzing a few bonds before dissociation. In contrast, exocellulases, also called cellobiohydrolases, have an active site tunnel and can only access the ends of a cellulose molecule [5] cleaving off cellobiose processively. There are several informative and detailed reviews on various aspects of cellulases [2, 6–9]. This paper is an overview of the remarkable variety of cellulases and discusses the similarities and differences in their properties.

1.1 Eukaryotic and Prokaryotic Cellulases

Eukaryotic cellulases have been found in insects, plants and fungi while bacteria producing prokaryotic cellulases are found wherever cellulose is present such as in compost piles, soil, rotting wood, etc. Many animals, including ruminants, utilize the cellulose present in their food; however, they do not produce cellulases but rely on cellulolytic microorganisms, primarily bacteria, to hydrolyze the cellulose [10]. The rumen is an extremely anaerobic environment so all rumen organisms are strict anaerobes. Some insects, for example termites and wood roaches, degrade cellulose. At first it was thought that insects did not produce cellulases and that symbiotic cellulolytic bacteria, fungi or protozoa produced the cellulases they used for cellulose degradation. However, there is growing evidence that several different species of insects produce cellulases and thus contain cellulase genes. Insect cellulases have been isolated from four species of termites, *Macrotermes subhyalinus*, *M. michaelseni*, *Eoptotermes lactens* and

Natsutitermes walheri, as well as from the wood roach, *Panesthia cribrata* [11]. A gene encoding a family 9 endocellulase has been cloned from the termite *Reticulitermes speratus*, which contains a 450 base intron, proving it is a eukaryotic gene [12].

Cellulases are also produced by plants and participate in leaf and flower abscission, the ripening of fruits, as well as differentiation of vascular tissue and plant cell wall growth [13]. A number of plant cellulase genes have been cloned and sequenced. All of them belong to cellulase family 9 and none of them have been shown to contain a cellulose-binding domain (CBD) [14, 15]. Both of these results are surprising since the presence of a CBD appears to be important for degrading crystalline cellulose and most cellulolytic organisms contain cellulase genes from several cellulase families. The methods used to clone plant cellulases should have detected enzymes from any family. Most plants contain multiple cellulase genes and they appear to be regulated in different ways. The cellulases that are involved in fruit ripening are often induced by ethylene, while the cellulases involved in cell growth are often induced by auxin [16, 17].

In addition, a new class of proteins (α-expansins) has been discovered in plants that appear to disrupt the interactions between cellulose chains, without hydrolytic activity, to allow cell wall expansion [18]. A different, but related, set of molecules (β-expansins) has been found in pollen [19]. These molecules appear to help the growing pollen tube penetrate through the cell walls in the ovule allowing fertilization. There is preliminary evidence that the addition of expansin to cellulases stimulates crystalline cellulose hydrolysis [20].

Endocellulases have been isolated from plant pathogenic nematodes and four structural genes were cloned from two species [21]. All of the cellulase catalytic domains belong to family 5 and two of them also code for family II CBDs. The enzymes show 37% identity in their amino acid sequence to several bacterial cellulases.

1.2 Anaerobic Versus Aerobic Cellulases

There are two quite different ways that cellulolytic bacteria and fungi deal with the problem raised by the insolubility of cellulose and their inability to ingest cellulose particles. Most anaerobic microorganisms produce multienzyme complexes, called cellulosomes, on their cell surface while most aerobic microorganisms secrete a set of individual cellulases into the external milieu where the enzymes act synergistically to degrade crystalline cellulose. In each case, the products of digestion are oligosaccharides, mostly cellobiose and glucose, that are transported into the cell and metabolized. Some aerobic fungi also secrete cellobiase so that glucose is the major end product of cellulose degradation.

One possible explanation for the difference in cellulase organization is that anaerobic organisms are more energy limited than aerobic organisms, and thus it is more important for them to retain the products of cellulose digestion. Some anaerobic organisms are tightly bound to cellulose by their surface cellulases so that the products of digestion are released in a confined space between the insoluble cellulose and the organism. With free cellulases, the hydrolysis products

are in solution and would be more available to competing organisms. Since there is an anaerobic bacterium, *Clostridium papyrosolvens* C7, which secretes cellulase complexes into the medium and thus would not be able to retain all of the digestion products, there may be other advantages of complex formation [22, 23].

2 Cellulase Domains

Cellulases usually have several domains. All of them contain one catalytic domain and a few with multi-catalytic domains have been found. A lambda recombinant from a genomic library of *Caldocellum saccharolyticum* encoded three multi-catalytic domain enzymes: CelA with family 9 and family 48 cellulase catalytic domains, ManA with β-mannanase and endocellulase catalytic domains, and CelB with xylanase and endocellulase catalytic domains [24–26]. *Anaerocellum thermophilum* CelA has both a family 9 and a family 48 catalytic domain [27]. *Clostridium thermocellum* CelJ has a family 5 endocellulase domain combined with a family 9 processive endocellulase domain [28]. There have not yet been detailed studies into whether the activity of a cellulase with two catalytic domains is higher than the activity of a mixture of enzymes containing the two domains by themselves. There is a report that an enzyme containing a family 5 domain and a *Bacillus* 1–3, β1–4 glucanase domain has higher activity on β-glucan than either of the domains alone [29].

After catalytic domains, the next most common domains are CBDs which are usually joined to the catalytic domain by a short linker peptide. Cellulases that are present in cellulosomes contain short domains called dockerins that bind to specific sites on a scaffoldin protein to form a cellulosome [30]. A number of cellulases contain fibronectin-like domains, but the function of these domains is not known [6]. There are several other domains with unknown functions [6].

2.1 Catalytic Domain Families

Many cellulase genes have been cloned and sequenced. At this time, there are at least 112 sequences reported in the Swiss protein data base. Henrissat and colleagues have grouped the bulk of these genes into eleven families (5–9, 12, 44, 45, 48, 60, 61) based on both sequence homology and hydrophobic cluster analysis [31–34]. There is a web site at http://expasy.hcuge.ch/cgi-bin/lists?glycosid.txt which contains current information on all glycosyl hydrolase families including cellulases. In addition, several cellulase genes code for enzymes that do not resemble any other known cellulases. The presence of a large number of cellulase families is unusual as most enzymes have only a few families. This heterogeneity presumably results from the abundance of cellulose, the complexity and variability of plant cell walls, which are the actual substrates of most cellulases, and the difficulty of degrading plant cell wall cellulose.

X-ray structures have been determined for more than a dozen cellulases from eight different families. The results support the idea that all of the cellulases in a given family have the same basic structure [35–49]. Gideon Davies has

written a concise overview of the basic structures [50]. At least six completely different folds can lead to an active cellulase. Some cellulase families include both exocellulase and endocellulase genes, while others contain only one type of enzyme. Despite the dramatic difference in the way their cellulases are organized (free versus bound), the families to which the catalytic domains of aerobic and anaerobic cellulases belong show a great deal of overlap. Furthermore, there is some overlap between the families to which bacterial and fungal cellulases belong, although there are families with cellulases from only one class of organism. The one property that is completely conserved in all members of a family is the stereochemistry of cleavage (retaining or inverting) of the cellulose β1–4 bond (Fig. 1) [2, 51, 52] which is discussed in Sect. 5.

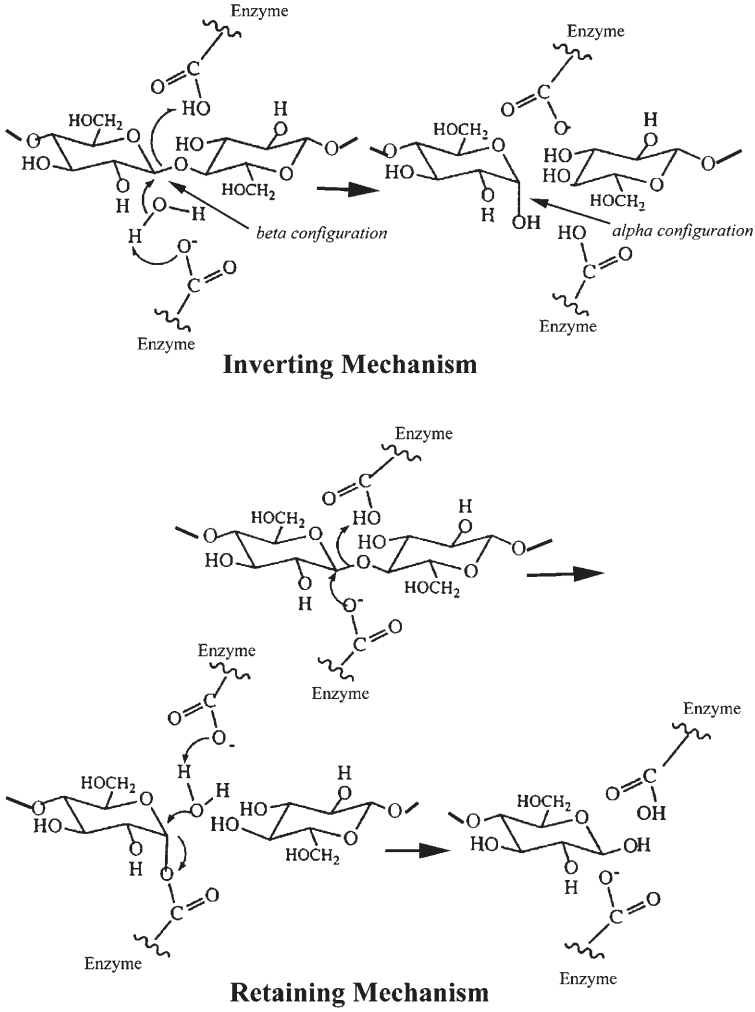

Fig. 1. The two stereochemically different mechanisms of hydrolysis for cellulases

Family 5 is the largest cellulase family containing 57 genes. Of these 52 code for endocellulases and they are retaining enzymes. The other five genes code for β1–3 exoglucanases. Most of the family 5 genes are from bacteria, but some are from fungi. The three-dimensional structures of four family 5 catalytic domains have been reported and they have an α/β-barrel fold, which is the most common fold found among all proteins [40, 44, 53, 54]. Many of the family 5 genes do not code for a cellulose-binding domain. Those that also lack a dockerin domain may not function in the degradation of crystalline cellulose. This has been shown to be true for the carboxymethylcellulase (CMCase) from the anaerobic bacterium *Prevotella bryantii*, where the gene appears to be required for growth on β-glucan, a glucose polymer with alternating β1–4,1–3 linkages [55].

Family 6 contains nine genes coding for both endo- and exocellulases from bacteria and fungi. The structures of two family 6 enzymes, *Trichoderma reesei* CBHII – a fungal exocellulase and *Thermomonospora fusca* E2 – an actinomycete endocellulase, have been determined and they are modified α/β barrels that close the barrel in a slightly different way than it is closed in standard α/β-barrel proteins [43, 46]. When the three-dimensional structures of CBHII and E2 are overlaid there are two loops which cover the active site cleft to form an active site tunnel in CBHII [43]. The E2 active site cleft is much more open because one loop is much shorter and the other has a different conformation. The enzymes in this family all catalyze hydrolysis with inversion of the anomeric carbon configuration.

Family 7 contains only fungal genes, coding for both endo- and exocellulases. The enzymes in this family all utilize the retaining mechanism. The structure of the *T. reesei* CBHI catalytic domain has been determined and it is a unique structure with a β sandwich forming the active site and many loops connecting the β strands [38, 39]. This structure is larger than those of the family 5 and 6 catalytic domains and has a long active site tunnel with enough room for seven glucosyl residues. Three more family 7 structures have been solved: *Fusarium oxysporum* endoglucanase I with a nonhydrolyzable thiooligosaccharide substrate analogue [56], *Humicola insolens* endoglucanase I [57], and *T. reesei* endoglucanase I [58].

Family 8 contains nine genes, all bacterial, which appear to code for endocellulases utilizing the inverting mechanism. The structure of a family 8 endocellulase, *C. thermocellum* Cel A, has been determined and it is an $(\alpha/\alpha)_6$ barrel similar to those found in families 9 and 48 [35, 59].

Family 9 contains 19 cellulase genes that belong to two subfamilies distinguished by the presence or absence of a family III CBD closely attached to the catalytic domain. All of the enzymes in this family are inverting. The three-dimensional structures of the catalytic domain of *C. thermocellum* CelD [41] and the catalytic domain plus the family III CBD of *T. fusca* E_4 [45] have been determined and the catalytic domains are $(\alpha/\alpha)_6$ barrels. The E4 family III CBD was aligned with the catalytic cleft so that a cellulose molecule bound in the active site could also be bound to the CBD. The enzymes without the attached CBD are all endocellulases, while E4, with the attached CBD, is a processive endoglucanase [41, 60]. This family does not contain any fungal genes, but includes genes from both bacteria and plants.

Family 12 contains nine genes, all coding for retaining endoglucanases. They include bacterial and fungal genes. The structure of an endocellulase has been determined and it is a jelly roll made up of β-sheets very similar to the structure of a family 11 xylanase [47].

Families 44, 60 and 61 are small cellulase containing families with only a few members thus far. Family 44 includes an inverting endoglucanase and a mannanase from bacteria. No structures have been determined for these families.

Family 45 contains five endocellulases from bacteria and fungi. The structure of *Humicola insolens* endoglucanase V has been solved and consists of a six-stranded β-barrel domain with a long open groove across the surface. This enzyme catalyzes hydrolysis with inversion at the anomeric carbon atom [36, 37, 61].

Family 48 contains six cellulase genes and they all code for inverting enzymes. Some of the enzymes studied so far appear to have low specific activities on cellulose substrates and several are present in their respective organisms in relatively large amounts suggesting they are exocellulases [25, 27, 62–66]. These genes are present in both anaerobic and aerobic bacteria as well as anaerobic fungi. The three-dimensional structure of one family 48 catalytic domain, *C. cellulolyticum* CelF, has been reported and it is an $(\alpha/\alpha)_6$ barrel similar to that found for family 8 and family 9 cellulases [67]. As expected for an exocellulase part of the active site is in a tunnel.

2.2 Cellulose-Binding Domains

Most anaerobic cellulases either have no cellulose-binding domain (CBD) or, as in *C. thermocellum* cellulosomes, the CBD is attached to the scaffoldin molecule which is in turn attached to multiple catalytic domains. Many aerobic organisms make cellulases with a CBD attached to the catalytic domain via a flexible linker which is often glycosylated. The CBD is usually found at either the N- or the C-terminus in nearly equal numbers. So far, 13 different cellulose-binding domain families have been reported based on sequence differences [6, 68, 69]. Family I CBDs are found only in fungi and are 33–36 amino acids long. Family II CBDs are found only in bacteria and are about 100 residues long. Removal of family II CBDs from *T. fusca* cellulases reduces their activity on crystalline cellulose severely, but affects activity on more soluble or amorphous substrates such as carboxymethyl cellulose (CMC) and phosphoric acid swollen cellulose (SC) much less [70–72]. This has also been shown for removal of the fungal family I CBD from *T. reesei* CBHI and CBHII [73]. Some xylanases contain family II CBDs that can bind to xylan as well as cellulose, but most CBDs do not appear to bind xylan [74, 75]. Family IIIa CBDs are known to anchor the *Clostridium* scaffoldin strongly to cellulose [30]. The *T. fusca* E4 family IIIc CBD has been shown to facilitate processivity of the cellulose molecule through the catalytic active site but does not bind tightly to cellulose [60]. The family IV CBD from *C. fimi* CenC has been shown to bind only to SC and not to crystalline cellulose and has a binding cleft rather than a binding face, enabling it to bind to single cellulose molecules [68, 76].

Seven CBD structures have been determined: the nuclear magnetic resonance (NMR) structure of the family I CBD of *T. reesei* [77], the small angle X-ray scattering structure of the family II *C. fimi* CenA CBD [78], the NMR structure of the family II *C. fimi* Cex CBD [79], the crystal structure of the family IIIc internal CBD together with the catalytic domain of *T. fusca* E4 [45], the crystal structure of the *Clostridium* family IIIa CipA CBD [48], the NMR structure of the family IV CBD of *Cellulomonas fimi* CenC [76], and the NMR structure of the family V CBD of *Erwinia chrysanthemi* EGZ [80]. Although the structures of families I, II and III differ, they are all made up basically of β-sheets and have a flat face containing several aromatic and potential hydrogen-binding residues that are spaced so they would be able to stack along glucose residues in a cellulose crystal. Tormo et al. have presented models comparing the interaction of families I, II and III CBDs with cellulose [48].

The primary role of a CBD appears to be to attach the cellulase to cellulose which is generally accepted as increasing the effective concentration of the cellulase and increasing the time the enzyme is close to its substrate. A CBD has been reported to disrupt the structure of cellulose [81], but this has not been found in most cases [82]. Using fluorescence recovery after photobleaching, it has been shown that *Pseudomonas* CBDs were able to move along the surface of cellulose without dissociating and that 70% of the bound molecules were mobile [83]. Studies of the binding of cellulases to cellulose show that sometimes binding is fully reversible but in many cases binding appears to be partially or even totally irreversible [68, 84–87]. Although CBDs have many features in common, there is mounting evidence from sequence comparisons [60] and mutagenesis experiments [88, 89] in combination with binding assays that small differences in amino acids dramatically affect binding to cellulose and thus the function of a particular CBD.

2.3 Cellulosome Structure

The best studied anaerobic cellulolytic microorganism is the thermophilic bacterium *C. thermocellum*, which contains at least 24 different cellulase genes [7, 90]. Most of the genes code for cellulases that are present in cellulosomes, large complexes of cellulases, usually found on the cell surface; however, several code for cellulases that appear to be secreted as individual enzymes and contain cellulose-binding domains [91]. In addition to cellulases, there are several xylanases present in cellulosomes. A detailed summary of the current knowledge on the structures of the cellulosome components has been written by Bayer et al. [30].

A key component of cellulosomes is a 200,000 MW protein, scaffoldin, that contains at least nine copies of a cohesin domain, a family IIIa cellulose-binding domain, and a dockerin-like domain that appears to bind scaffoldin to the cell surface. The cohesin domains are the binding sites for the dockerin domains that are present at the C-terminus of each cellulase molecule present in the cellulosome. At this time, it appears that all the cohesin domains of a given species bind with nearly equal affinity to the dockerin domains present on the cellulosomal enzymes of that species [92]. This result suggests that cellulosomes are a

complex mixture of different sets of enzymes although there is no direct experimental data on the composition of cellulosomes. Calcium is required for the assembly of the cellulosome [92] and cell-free cellulosomes can be dissociated by incubation at 60 °C in the presence of ethylenediaminetetraacetic acid (EDTA) and cellulose [93]. The family IIIa cellulose-binding domain on the scaffoldin binds very tightly to cellulose and the cellulosome undergoes a conformational change when bound to cellulose [93].

On average, a single scaffoldin molecule and its attached enzymes would have a molecular weight of about one megadalton. There is a wide range of different cellulosome sizes (0.5 to 50 megadaltons) and it is not clear how the individual scaffoldin complexes are joined to form polycellulosomes [94]. Three molecules have been identified on the *C. thermocellum* cell surface that contain cohesin-like domains, SdbA, Orf2p and OlpB. These proteins bind selectively to the dockerin-like domain present in scaffoldin and contain, respectively, a single cohesin-like domain, two and four domains [30].

Cellulosomes have been studied in three mesophillic *Clostridial* species, *C. cellulovorans* [95], *C. cellulolyticum* [96] and *C. papyrosolvens* [22, 23] There are differences in the detailed structures of the scaffold proteins, but the basic features (described above) are similar. Some other anaerobic bacteria appear to use different mechanisms to attach their cellulases to the cell surface, but the details of these mechanisms are not known [10]. *C. papyrosolvens* makes cellulosomes that are released into the culture supernatant and do not bind tightly to cellulose. These complexes can be fractionated by ion-exchange chromatography into at least seven fractions with molecular weights from 500 to 650 kDa. The complexes differ in activity and show synergy in the hydrolysis of crystalline cellulose [22]. *Neocallimastix frontalis*, an anaerobic rumen fungus, appears to produce cellulosomes [97, 98], but there are differences from *Clostridial* cellulosomes in the specific enzymes present in the complexes and the sequence of a scaffoldin protein has not been determined.

A comparison of a cell-free preparation of *C. thermocellum* cellulosomes with a *T. reesei* cellulase mixture found that the cellulosome preparation could extensively solubilize (>80%) cotton at a faster rate. On the other hand, the *T. reesei* mixture had a higher rate on filter paper and a slightly better rate on Avicel (powdered crystalline cellulose) [99]. A major unsolved question is how the attachment of cellulases to scaffoldin creates the observed high cellulase activity. The large size of cellulosomes would prevent them from accessing the many pores in cellulose. In sieved Avicel with an average diameter of 100 μm, more than 90% of the surface that is accessible to free cellulases is present in pores [100]. Somehow the presence of multiple cellulases on the cellulosome or their organization is able to compensate for the inability of the cellulosomes to attack the cellulose in the pores. Possibly, the arrangement of the different classes of cellulases in close proximity allows the enzymes to synergistically attack cellulose from the surface of the microfibril. One other reason for the high activity may be the presence of the family IIIa CBD on scaffoldin, which binds the cellulosome tightly to cellulose.

There does not appear to be a special class of cellulases in cellulosomes since most of the cellulosomal hydrolases belong to the same set of families as those

of free cellulases. *C. thermocellum* cellulosomes contain three different endocellulases in family 5, one in family 8, one in family 26 and two in family 9. In addition, there are two different processive endoglucanases that have family 9 catalytic domains joined to a family III CBD and two family 48 exocellulases. From what is known about other cellulase systems, it would be expected that cellulosomes would contain at least one exocellulase that attacks the nonreducing end of cellulose molecules, but so far one has not been identified. This may be because exocellulase genes are very difficult to identify by activity screens, which is how most *C. thermocellum* genes were cloned.

It is interesting that anaerobic ruminal fungi cellulosomes also contain a family 48 exocellulase, rather than the family 7 *T. reesei* CBH I type that is produced by aerobic fungi [98]. Both of these attack the reducing ends of cellulose molecules, but CBH I appears to be more active in synergistic mixtures than the family 48 enzymes. In the case of *T. fusca*, which also produces a family 48 exocellulase, the addition of CBH I to *T. fusca* crude cellulase nearly doubles its specific activity on filter paper. Bacteria may lack a CBHI-like enzyme for evolutionary reasons or because it cannot be expressed in prokaryotes, but these reasons do not apply to anaerobic fungi since they contain family 1 CBDs similar to those present in aerobic fungi.

2.4 Linkers

Linker*s* are short amino acid sequences that connect the different domains found in cellulases. Often they are rich in proline and threonine such as the PTPTPTPTT(PT)7 of *C. fimi* CenA [101] and the NPSGGNPPGGNPPGTTTTRRPATTTGSSPGP of *T. reesei* CBHI [102], but sequences such as PEEGEEPGGGEGPGGGEEPG in *T. fusca* E4 have also been found [103]. The Thr and Ser residues of fungal linkers are often O-glycosylated and some bacterial enzymes have been found to be lightly glycosylated [71]. Proline does not have a free amino group to participate in the peptide bond and when it is present in the amino acid chain regular hydrogen bonding is interrupted preventing the formation of α-helices and β-sheets. Glycine, on the other hand, has no side chain and has unique conformational flexibility. Thus, depending on their composition, linkers with prolines would be expected to have an extended form and linkers with many glycine residues are likely to be flexible. Thus far, there have not been any crystal structures solved of cellulases with linkers attached.

Removal of the first one third of the *T. reesei* CBHI linker reduced the binding of the enzyme to cellulose at high enzyme concentrations but did not affect the enzymatic activity on crystalline cellulose; removal of all of the linker did not affect binding but greatly reduced activity [102]. Deletion of the *C. fimi* CenA linker appeared to change the relative orientation of the catalytic domain and the binding domain as seen from small-angle X-ray scattering studies, and the catalytic efficiency on Avicel decreased by 50% [101]. The absorption properties stayed the same but the enzyme without a linker could no longer be desorbed by water. There are no glycines in that particular linker and it may well have a more rigid conformation. The ability of an enzyme to "desorb" or "move"

along a cellulose microfibril could be an important property for maximum activity.

Linkers are an easy target for protease activity but limited proteolysis of endocellulases is probably beneficial to the cellulase system because the more compact catalytic domains should be able to get into the smaller pores of crystalline cellulose. The function of glycosylation may be to protect against proteolysis. *T. fusca* E3 purified from an *E. coli* clone (not glycosylated) had the same activity as native glycosylated E3 [71].

3 Conservation of Cellulase Genes

There is a great deal of conservation among the cellulases of aerobic organisms. A set of organisms were isolated from both North and South America that could grow on cellulose at 55 °C. Antisera to five of the *T. fusca* cellulases that were tested all gave lines of identity on Ochterlony diffusion plates against all twelve isolates [104]. Furthermore, *T. curvata* supernatant not only gave lines of identity with the *T. fusca* enzymes on an Ochterlony diffusion plate, but the DNA from a *T. fusca* cloned cellulase gene and a *T. curvatae* clone appeared to be identical when tested for DNA duplex formation after they were melted and reannealed [105].

A comparison of the six cellulases produced by *T. fusca* with the six produced by the mesophillic soil bacterium *C. fimi* [72, 106] also shows a large amount of conservation. Both organisms produce two exocellulases, three endocellulases and a processive endoglucanase. Furthermore, the catalytic domains of each type of cellulase are in the same family so that the organisms essentially produce identical sets of cellulases. Despite their similarities, it is clear that the two sets of cellulase genes did not come from a common ancestor, but must have resulted from convergent evolution. The percent identity between the pairs of cellulase catalytic domains ranges from a low of 24% to a high of 80%. Finally, the location of the CBDs (N- or C-terminal) differs between four of the five pairs of enzymes that have CBDs (Table 1).

This cellulase conservation is also seen with most aerobic fungi; for example, the sets of cellulases produced by brown rot and white rot fungi have been shown to be similar [107]. The set of enzymes produced by aerobic fungi are not

Table 1. Relationship between *T. fusca* and *C. fimi* hydrolases

Catalytic domains:						
Family	9	6	6	9	5	48
T. fusca	E1	E2	E3	E4	E5	E6
C. fimi	CenC	EgA	CBHA	CenB	CenD	CBHB
% Identity	59	42	55	80	21	33
Binding domain locations:						
T. fusca	C	C	N	C	N	N
C. fimi	–	N	C	C	C	C

that different from those produced by aerobic bacteria, although the fungi do not seem to produce a processive endoglucanase and the bacteria do not seem to make any family 7 enzymes.

An unusual feature of some anaerobic bacteria is the extensive variability in the cellulase genes present in different isolates of a given species. In the case of the rumen bacterium *Ruminiccoccus albus*, there was no cross hybridization between two cellulose genes from two organisms isolated from different cows in the same herd [108]. Furthermore, while six sequenced *R. albus* cellulase genes from six different isolates all belong to cellulase family 5, most of them are more similar to cellulases from other bacteria than to each other. At this time, it is not clear why there is such a large difference in this property between the aerobic and the anaerobic organisms. It is possible that it results in some way from the nature of the cellulosomal versus free cellulases produced by each type of organism.

4 Multiple Cellulases

A major question in cellulase research is why multiple cellulases are produced when there is only one type of bond (β1–4 linkage) present in cellulose. An interesting example of multiple cellulases is *T. fusca*. The reason for two different exocellulases is that E_6 attacks the reducing end of cellulose, while E_3 attacks the non-reducing end [109]. Furthermore, two exocellulases give synergism if, and only if, they have opposite specificities [70]. Most known cellulolytic organisms produce two exocellulases with different specificities. *T. fusca* produces an enzyme, E_4, that belongs to a new class of cellulases, called processive endoglucanases [60]. E_4 can give synergism with every other type of cellulase; i.e. with endocellulases and both classes of exocellulase. Furthermore, it can increase the synergy when it is added to a mixture containing a member of each class of exocellulase and an endocellulase. Thus it is possible to explain the reason for four of the six *T. fusca* cellulases based on their ability to increase the rate of hydrolysis of crystalline cellulose. Cellulase E_1 has the lowest activity on crystalline cellulose of all six enzymes, but the highest activity on soluble substrates (CMC and oligosaccharides). It seems likely that E_1 is not important for the hydrolysis of crystalline cellulose, but functions in the hydrolysis of soluble or amorphous substrates. Either E_2 or E_5 can function with equal effectiveness as an endoglucanase in mixtures degrading crystalline cellulose. However, the activities of these enzymes on a number of cellulosic substrates are quite different, so they may well hydrolyze different plant cell wall materials at significantly different rates. Therefore, the different cellulases have different properties that allow each of them to function differently in plant cell wall degradation. Further evidence for different roles for the different *T. fusca* cellulases with different substrates is the fact that, although they are all induced by cellobiose and repressed by good carbon sources, they are not regulated coordinately [65].

Another interesting finding is that both bacterial and fungal aerobic cellulolytic organisms appear to produce much more exocellulase than endocellulase. For *T. reesei*, the exocellulases CBHI and CBHII make up about 80% of the total

cellulase protein [5], while for *T. fusca* growing on cellulose, the exocellulases E_3 and E_6 make up 73% of the total cellulase protein [65]. Since the average cellulose chain is longer than 1000 residues, there are many more potential sites of attack for endocellulases than for exocellulases on a free chain. However, in crystalline cellulose, most glucose residues in cellulose molecules are hydrogen bonded to neighboring chains; it is likely that end residues are easier to dissociate since they have hydrogen-bonded neighbors on only one side. Once an exocellulase has begun to processively hydrolyze a cellulose chain end, it must continue to pull the chain away from its neighbors as it proceeds. This difficult task would be slower than that of an endocellulase which is to hydrolyze the middle of an available chain and then dissociate. This could be a possible reason why more exocellulases are produced in cellulolytic systems.

4.1 Cellulase Synergism

Synergism refers to the ability of a mixture of cellulases to have higher activity than the sum of the activities of the individual enzymes. Individual cellulases alone will not degrade crystalline cellulose beyond about 5%, no matter how much enzyme is added to the reaction. From the currently available data it can be postulated that synergism in crystalline cellulose hydrolysis occurs when two or more cellulases attack different sites in cellulose and, as a result of their attack, new sites are created for each other. For example, when a non-reducing end attacking exocellulase binds and processively cleaves a cellulose chain, it disrupts the hydrogen bonds to neighboring chains creating some new sites at which endocellulases can attack; one or more reducing ends may also be exposed during this process. Thus, this exocellulase can synergize with both endocellulases and reducing end attacking exocellulases. Endocellulases create both types of ends with each cleavage so they can synergize with both types of exocellulase. It has been shown experimentally in synergistic mixtures containing an endo- and an exocellulase that hydrolysis by the endoglucanase is stimulated as much as hydrolysis by the exocellulase [70]. This model for synergism requires no interaction between the cellulases. This is consistent with the finding that cellulases from unrelated organisms, which are not likely to have sites that will interact, often give as much synergism as cellulases from the same organism. It is also consistent with a study in which pretreatment of cellulose with an endocellulase increased its rate of degradation by an exocellulase [110].

4.2 Fragmentation Activity

In addition to cleaving individual cellulose chains into small oligosaccharides, cellulases can also cleave cellulose particles into smaller particles. This activity is called fragmentation or short fiber formation. Quantitative studies of fragmentation utilized uniform sieved Avicel particles as the substrate and a particle counter to measure the number and size of the particles produced [111, 112]. Fragmentation activity could only be shown with particles larger than

1,000 Å in diameter [112]. The kinetics of fragmentation differ from the kinetics of the standard reducing sugar production assay in that fragmentation is linear with both time and enzyme concentration. Cellulases also showed synergism in fragmentation, but the relative activities (reducing sugar production and fragmentation) of enzyme mixtures in the two assays were often different [111]. At this time, it is not clear how cellulases cause fragmentation and why fragmentation has different kinetics than reducing sugar production.

5 Mechanisms of Cellulase Activity

There are two stereochemically different mechanisms of hydrolysis for cellulases as shown in Fig. 1: hydrolysis with inversion and hydrolysis with retention of the β-conformation of the cleaved bond. Enzymes that utilize the retaining mechanism are usually able to carry out transglycosylation, while inverting enzymes can not. Two carboxyl side chains play key roles in both mechanisms. In inverting enzymes, one carboxyl side chain is protonated and acts as a catalytic acid, donating its proton to the glycosidic oxygen of the leaving group. The other carboxyl side chain acts as a catalytic base removing a proton from the water molecule which attacks the C_1 carbon thus inverting the linkage from β to α. In a retaining enzyme, the catalytic base attacks the C_1 carbon forming a covalent intermediate with an inverted glycosidic bond, while the catalytic acid again donates a proton to the glycosidic oxygen of the leaving group. In a second step, a water molecule attacks the C_1 carbon again inverting the linkage leading to an overall retention of configuration.

There is strong evidence from chemical modification, site-directed mutagenesis, sequence alignments of families, and structural studies for the involvement of carboxyl side chains in the active site of all glycosyl hydrolases that have been studied. An interesting finding is that, for inverting enzymes, the distance between the potential catalytic acid and base side chains averages around 9.5 Å, while for the retaining enzymes it is about 5 Å [113].

There are four Asp residues that are conserved in family 6 (inverting) cellulases which have been studied mechanistically using mutants of *T. reesei* CBHII [114], *T. fusca* E2 [115] and *C. fimi* CenA [116]. All four Asps are present near the site of bond cleavage in the *T. reesei* CBHII structure [43] but only three are structurally conserved in *T. fusca* E2 [46]. However, there is strong evidence from site-directed mutagenesis data that the loop carrying the structurally nonconserved Asp79 in E2 can move, and it seems likely that after the substrate binds, the Asp residue is in a similar location to what it is in CBHII [117]. One of the conserved residues (equivalent to E2 Asp117) clearly functions as a catalytic acid in all three proteins. Another conserved residue (equivalent to E2 Asp156) is buried but is close to the catalytic acid and functions to shift the p*K* of the catalytic acid [114–116]. The role of the other two residues is less clear. There is evidence that *C. fimi* CenA Asp392 (equivalent to E2 Asp265) functions as the catalytic base [116], but when this residue was mutated in E2 it had reduced activity but not the orders of magnitude reduction expected [115]. It also had greatly reduced binding [118] which correlated with the reduced activity. In

T. fusca E2, this residue appears to be important for substrate binding but it is not the catalytic base. The fourth conserved residue E2 Asp79 was also mutated and found to be important for catalysis but it is not clear exactly what its role is.

6 Cellulase Regulation

Many celluloytic organisms use two different mechanisms to regulate cellulase synthesis: induction by cellobiose and repression by any good carbon source. This is the case for *T. fusca*, where all six cellulases and one xylanase appear to be regulated by both of these controls [119–121]. The regulated genes are not linked, but all of them contain one or more copies of an inverted repeat sequence, TGGGAGCGCTCCCA, upstream of the translation start site. This sequence has been shown to be the binding site for a cytoplasmic protein that has been purified and its N-terminal sequence shows strong homology to members of the *lac* repressor gene family. This sequence has been found next to most actinomycete cellulase genes and in *Streptomyces reticuli* it has also been shown to be the binding site for a repressor [122].

The levels of all six *T. fusca* cellulases have been measured in cultures grown on six different carbon sources and they are not coordinately regulated, although they all respond to both induction and repression [65]. Both induction and repression regulate transcription of *T. fusca* endocellulase E5 [123]. The induction mechanism appears to be complicated as the repressor protein is constitutively expressed, but repressor binding activity can only be detected in extracts from induced cells. Cellobiose inhibits repressor binding at concentrations above 10^{-4} M and it is the only sugar tested that strongly inhibits binding [119]. This finding confirms the proposal that cellobiose released by the action of a low level of constitutive cellulose is the actual inducer for cultures grown on cellulose [120]. Very little is known about the mechanism of repression in *T. fusca*. Work on *T. curvatae* suggests that cyclic adenine 5′-monophosphate (AMP) might play a role [124], but this does not seem to be true for *T. fusca* [120].

There have been a number of studies of the regulation of *T. reesei* cellulase synthesis and a recent report describes major advances in our understanding of this system [125]. Sophorose (2-*O*-β-D-glucopyranosyl-α-D-glucose) or cellulose were found to be the best inducers in *T. reesei* and induction increased the level of cellulase mRNAs. There is no detectable cellulase mRNA in uninduced cells while there is at least a 1000-fold increase in cells grown on sophorose or cellulose. Glucose is a repressor that can completely block cellulase mRNA synthesis when added to sophorose- or cellulose-induced cultures. When a glucose-grown culture uses up all of the glucose, cellulase mRNAs are produced at about 10% the level of fully induced cultures [126]. An analysis of the *T. reesei* CBHI promoter found many binding sites for the fungal glucose repressor, CREI, some as far as 1800 bases upstream of the open reading frame, but the main cluster was about 700 bases upstream [127]. Sophorose induction only required 30 upstream bases. A *cre1* gene was isolated from *T. reesei* using polymerase chain reaction with oligonucleotide primers that are complementary to

conserved regions of *Aspergillus* CREA [128]. In addition, it was shown that this gene is inactivated in the hypercellulolytic *T. reesei* strain Rut-C30. Two *T. reesei* genes that coded for proteins that could activate the CBHI promoter in yeast were isolated by a yeast reporter gene expression approach. The isolated genes coded for two proteins, ACEI and ACEII, which contain zinc finger nucleic acid binding sequences but are otherwise not similar to any other proteins in the protein data bases [125]. At this time, it is not known why there are two different proteins or how they function in induction control.

7 Application of Cellulases

After proteases and amylases, cellulases are the third most important industrial enzymes in dollar value. They are currently added to detergents to remove damaged fibers (piles or fuzz) from cotton fabrics since dirt binds more tightly to the piles. They are also used for "biostoning" to replace the stone washing process for denim fabric as well as to change the texture of other cotton fabrics. They are added to some animal forage and feeds to increase digestibility and used to improve the extraction of juice from fruits and vegetables. In addition they are used to lower energy consumption in mechanical pulp production by improving the beatability, drainage and solubility of chemical pulps. A major potential application for cellulases is in the hydrolysis of biomass cellulose for the production of ethanol as a renewable liquid fuel. Himmel and co-workers at the National Renewable Energy Laboratory of the Department of Energy are trying to develop improved cellulases for biomass hydrolysis [129].

A problem in testing enzymes for industrial processes is the large amount of enzyme needed for trials. Detergent testing is usually done in a washing machine, while papermaking, even for hand sheets, requires fairly large amounts of enzymes. At this time, it has not been possible to correlate the results of simple cellulase assays with the results of testing for specific applications. Once we understand exactly how the enzymes change the substrates in an application, it should be possible to predict which enzyme or enzyme mixture will be best for a given application.

7.1 Engineering Cellulases

One long-term goal of cellulase research is to engineer mixtures of cellulases that have higher specific activities, greater thermostability and a wider pH profile in the hydrolysis of pretreated biomass than *T. reesei* crude extract which is currently the most active cellulase mixture known. It can be argued that cellulases have evolved with the highest possible activity on crystalline cellulose so that it will not be possible to design more active cellulases. However, the actual substrate that cellulases have evolved to degrade is not pure cellulose but plant cell walls, which are a complex mixture of cellulose, hemicellulose, lignin and other polymers such as mannan and pectin. Furthermore, since microorganisms cannot ingest particles, they have to release their cellulases outside the

cell so that the products of cellulose degradation are available to other organisms. Thus, organisms need to control the rate of hydrolysis to minimize the loss of degradation products to other organisms. Finally, the material that will be degraded in a biomass to ethanol process will not be pure cellulose, but the product from a specific pretreatment process. Thus, by determining the detailed molecular mechanism by which cellulases degrade cellulose, it should be possible to design a set of cellulases that are optimized to degrade the specific form of cellulose present in the pretreated biomass that will be used in the process.

In order to design better cellulases, it is necessary to determine the rate-limiting step in the hydrolysis of crystalline cellulose, since it is only by increasing the rate of this step that the rate of hydrolysis can be increased. Site-directed mutagenesis studies of *T. fusca* endoglucanase E_2 have shown that cleavage of the β1–4 linkage in crystalline cellulose is not the rate-limiting step. Mutants have been created that either increase or decrease the rate of bond cleavage, yet neither class of mutants changes dramatically the rate of filter paper hydrolysis, unless the rate of bond cleavage is reduced more than 10-fold [115]. The rate-limiting step is probably the binding of a cellulose molecule into the active site cleft. The next question is what E_2 residues or other cellulase residues participate directly in this step and how might the rate be increased. *T. reesei* CBHII Trp272 is a surface residue at the entrance to the active site that appears to be required for the rate-limiting step in crystalline cellulose hydrolysis. Ala and Asp mutants of this residue have dramatically lowered activity on crystalline cellulose with no major changes in binding or activity on swollen cellulose [130]. This residue is conserved in family 6 exocellulases but not in the endocellulases. A surface residue mutant of E2, Trp16Ile, caused a major change in substrate specificity, lowering swollen cellulose activity to 37% but maintaining activity on CMC, filter paper and BMCC at 69–85% [117]. A time course of swollen cellulose activity shows that the activity of the mutant totally stops after 1 h while the wild-type activity continues although at a slower rate. However, the time course on filter paper shows the mutant has the same pattern of activity as wild type [131]. This residue does not appear to be involved in the rate-limiting step for crystalline cellulose but seems to be important in coaxing the amorphous cellulose molecule into the active site of the enzyme.

Significant progress is being made in all areas of cellulase research and the determination of the three-dimensional structures of both catalytic and cellulose-binding domains is making a major impact on cellulase research. Despite the rapid progress there are still many unanswered questions. It is clear that while there are similarities between the sets of enzymes that different cellulolytic organisms use to degrade cellulose, there are also many significant and interesting differences.

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Reaction Kinetics, Molecular Action, and Mechanisms of Cellulolytic Proteins

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Cellulolytic proteins form a complex of enzymes that work together to depolymerize cellulose to the soluble products cellobiose and glucose. Fundamental studies on their molecular mechanisms have been facilitated by advances in molecular biology. These studies have shown homology between cellulases from different microorganisms, and common mechanisms between enzymes whose modes of action have sometimes been viewed as being different, as suggested by the distribution of soluble products. A more complete picture of the cellulolytic action of these proteins has emerged and combines the physical and chemical characteristics of solid cellulose substrates with the specialized structure and function of the cellulases that break it down. This chapter combines the fundamentals of cellulose structure with enzyme function in a manner that relates the cellulose binding and biochemical kinetics at the catalytic site of the proteins to the macroscopic behavior of cellulase enzyme systems.

Keywords. Cellulases, Cellulose, Hydrolysis, Model Mechanism, Structure, Kinetics

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1 Introduction

Cellulose, the most abundant biological compound on earth, is highly resistant to degradation. Fungi and bacteria that utilize this polymer as a carbon source have evolved a complex array of enzymes that de-crystallize and hydrolyze cellulose to liberate the individual glucose monomers. These enzymes are traditionally classified into three main groups that act synergistically to form a cellulolytic system. Enzyme kinetics, X-ray diffraction, NMR, and genetic sequencing data gathered over the last several decades have greatly illuminated the chemical mechanisms and the molecular structures that cellulolytic enzymes share and allow more precise classification and identification of these important enzymes.

Cellulolytic enzymes are being used in numerous industrial applications. Their utility in the generation of fermentative sugars is slowly increasing. The largest current industrial use of cellulolytic enzymes is in the textile industry. Cellulases are currently marketed as "biofinishing" agents for cellulose textiles such as denim, rayon, linen, and cotton. Cellulase treatment of these fabrics prevents fuzz and pill formation, increases smoothness and softness, and increases luster and color brightness [1].

Enzymatic action can be defined on three levels: operational kinetics, molecular architecture, and chemical mechanism. Operational kinetic data have given indirect information about cellulolytic enzyme mode of action along with important information useful for modeling cellulose hydrolysis by specific cellulolytic enzyme systems. These data are based on measurement of initial rates of enzyme hydrolysis with respect to purified celluloses and their water soluble derivatives over a range of concentrations of both substrate and products. The resulting kinetic patterns facilitate definition of the enzyme's mode of action, kinetic equations, and concentration based binding constants. Since these enable the enzymes' action to be defined with little direct knowledge of its mechanistic basis, the rate equations obtained are referred to as operational kinetics. The rate patterns have enabled mechanisms to be inferred, and these have often coincided with more direct observations of the enzyme's action on a molecular level [2–4].

Improved protein separation techniques utilizing liquid chromatography and electrophoresis coupled with X-ray diffraction and NMR studies have given insights into the three-dimensional structures of cellulolytic enzymes. This molecular architecture data coupled with DNA sequence information has given clues to the chemical mechanisms of enzymatic hydrolysis and molecular interaction between cellulose and the enzymes.

2 Enzyme Classification

Cellulolytic enzymes are classified into three main groups: cellobiohydrolases, endoglucanases, and β-glucosidases. According to the prevailing hypothesis, cellobiohydrolases (CBHs) attack the chain ends of cellulose polymers to re-

Table 1. Glycosyl hydrolase families

Family	Enzyme	Source
1	β-Glucosidase	*Clostridium thermocellum*
3	β-Glucosidase	*Trichoderma reesi*
3	β-Glucosidase	*Agrobacterium tumefaciens*
5	Endoglucanase B	*Clostridium cellulovorans*
5	Endoglucanase I	*Trichoderma reesi*
6	Cellobiohydrolase II	*Trichoderma reesi*
7	Cellobiohydrolase I	*Trichoderma reesi*
7	Cellobiohydrolase I	*Trichoderma viride*
9	Endoglucanase C	*Clostridium cellulovorans*
11	Xylanase	*Trichoderma reesi*
11	Xylanase	*Trichoderma viride*
13	α-Amylase	*Homo sapiens* (pancreas)
13	α-Amylase	*Bacillus subtilis*

lease cellobiose, the repeat unit of cellulose. Endoglucanases (EGs) decrease the degree of polymerization of cellulose by attacking amorphous regions of cellulose by random scission of the cellulose chains. β-Glucosidase completes the process by hydrolyzing cellobiose to glucose [5]. Cellulolytic systems have enzymes from all three groups. For example, the cellulolytic system from the filamentous fungus *Trichoderma reesei* contains two cellobiohydrolases, four endoglucanases, and one β-glucosidase [6].

With the advent of technology for identifying and sequencing genes for specific enzymes, another classification system has been proposed as an extension of the International Union of Biochemistry (IUB) Enzyme Nomenclature. The IUB nomenclature is based on the type of chemical reaction catalyzed by the enzyme. The proposed classification scheme adds a fifth number to the IUB classification indicating "families" [7]. These families are based on homology of primary amino acid sequence for all glycosyl hydrolases (EC 3.2.1.x). A difference of three standard deviations between a statistical comparison of two amino acid sequences was considered significantly different [7]. More than 45 families have been established from more than 480 sequenced glycosyl hydrolases with cellulolytic enzymes occupying eleven different families [8]. Table 1 lists several cellulolytic enzymes and a few other glycosyl hydrolases grouped by family. This system can be used as a way of comparing the sequence and thus the structures of different cellulolytic enzymes.

3
Characterization of Cellulosic Materials

Cellulose is a heterogeneous substrate that makes modeling cellulolytic enzyme hydrolysis difficult. Cellulose is composed of chains of glucose connected by β 1–4 glycosidic bonds. One chain end is termed the reducing end because the hemiacetal is able to open to expose the reducing aldehyde. The other chain end is called the non-reducing end because the 1 carbon in the hemiacetal is involv-

ed in the β 1–4 bond, preventing ring opening. Like starch chains, this gives cellulose chains directionality. Unlike starch, the repeat unit for cellulose is cellobiose, a glucose dimer, not glucose. The β 1 – 4 bond causes the glucose pyranose structure to align with alternating directionality. X-ray scattering patterns of pure crystalline cellulose from different sources have shown that, in cellulose crystals, chains are arranged in layered sheets. The chains within each sheet tend to align in a parallel fashion (as opposed to anti-parallel) and are linked by hydrogen bonds while they stack using van der Waals forces [9]. Crystalline cellulose can assume different forms. The most common are cellulose I and II. Cellulose I is the native form of cellulose found in cotton, ramie, wood, jute, and flax [10]. The cellulose chains are arranged so that the glucopyranose rings are parallel to the bc plane of the crystal (Fig. 1). This gives distinctly different characteristics between the two crystal faces with exposed glucopyranoside rings and the other faces. The binding of cellobiohydrolase and endoglucanase has been suggested to favor the faces with exposed glucopyranoside rings [11–13]. Highly conserved aromatic amino acid residues that can stack on the exposed rings have been noted between all enzyme binding domains, which are common structural features of carbohydrate binding proteins [14].

Cellulose II is a "swollen" form of cellulose made by alkali treatment of cellulose I [10]. The cellulose chains are rotated 30° from parallel to the ab crystal face (Fig. 2). Although this 2-chain cell model is correct for most celluloses, some require an 8-chain cell model. However, there is still a great deal of controversy over the nature of crystalline cellulose.

The two most dominating characteristics of cellulose are the specific surface area, SSA, and the crystallinity index, CrI. Specific surface area is defined as the amount of surface area per mass of cellulose. Crystallinty index is the relative amount of crystalline cellulose as opposed to the amount of amorphous cellulose [15]. In biomass, cellulose is closely associated with hemicellulose and lignin. Agricultural residues are composed of 30 – 40 % cellulose, 25 – 35 % hemi-

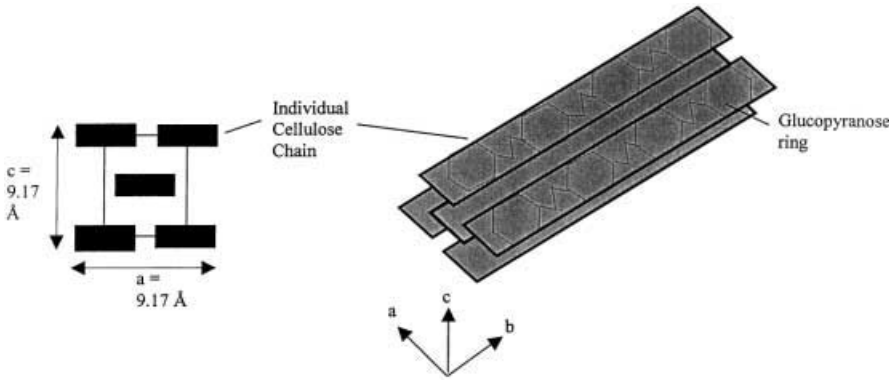

Fig. 1. Cellulose I crystal. X-ray crystallography in the 1950s solved the basic crystal unit of cellulose I. The axes of the repeat unit (cellobiose) are: a = 8.14 Å, b = 10.3 Å, and c = 9.17 Å. The faces of the glucopyranose rings are parallel to the ab plane of the crystal

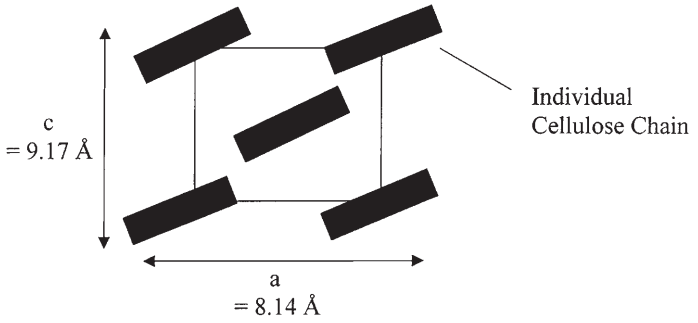

Fig. 2. The ac plane of cellulose II crystal. The b plane (not shown) is unchanged (10.3 Å) from cellulose I. However, the orientation of the cellulose chains has shifted from parallel to the ac plane by 30°

cellulose, and 10–15% lignin with the remaining percentage in protein, simple sugars, and minerals [5]. Cellulose is naturally semi-crystalline with regions of high crystallinity averaging approximately 200 glucose residues in length separated by amorphous regions. Due to the large influence of substrate characteristics on cellulose hydrolysis, most research in this area utilizes a more defined form of cellulose as the enzyme substrate. Bacterial micro-crystalline cellulose (BMCC) is the most completely characterized cellulose available. It is recovered as small particles from blue-green algae such as Valonia macrophysa or Acetobacter xylinum. These particles are nearly 100% crystalline cellulose. Due to the cost of bacterial micro-crystalline cellulose, micro-crystalline cellulose derived by acid hydrolysis of wood, such as Avicel (FMC Pharmaceuticals, Philadelphia, Pennsylvania, USA), is more commonly used. Avicel is produced by controlled acid hydrolysis under reflux conditions. Besides micro-crystalline cellulose, Solka Floc (Brown & Co, Berlin, New Hampshire, USA), a mixture of crystalline and amorphous cellulose produced by hammer-milling sulfite wood pulp, is also often used as a substrate for the study of cellulolytic enzyme kinetics. The specific surface area is 3.90 m^2/g and the crystallinity index is 77.4 for 270–400 mesh Solka Floc [15].

4
Operational Kinetics and Cellulose Binding Models

Although the Michaelis-Menten model accounts for the kinetic properties of many enzymes, one of the assumptions that this model is built upon does not hold for cellulase systems. Michaelis-Menten modeling assumes a homogeneous system where mass transfer (substrate-to-enzyme and product-from-enzyme) is not rate limiting, therefore only the catalytic step (enzyme-substrate complex to enzyme-product complex) is the governing rate of the reaction. However, cellulose hydrolysis occurs in a heterogeneous system of two phases. The enzymes are dissolved in an aqueous phase while the cellulose exists in a crystalline solid. To complicate modeling further, cellulose itself is a heteroge-

neous polymer that can assume different crystal forms and have varying overall degrees of crystallinity. These physical characteristics all affect hydrolysis rate. Adsorption of the enzyme from the aqueous phase to the solid surface has been shown not to affect significantly the overall rate of hydrolysis [15]. However, adsorption isotherms are important in determining the effective enzyme concentration at the cellulose surface. Therefore, modeling of enzymatic cellulose hydrolysis has been attempted over the years with emphasis on relating hydrolysis kinetics to enzyme adsorption. Although many models have been proposed and used for extrapolating expected conversions from one experimentally measured condition to the next, none are generally applicable for solid substrates. This is due to the heterogeneity of native cellulose, the difficulty in characterizing the important parameters of the substrate, and the complexity of each cellulolytic system in terms of number of different enzymes, synergistic effects, and product inhibition.

5 Development of Kinetic Models

The study of the kinetics of enzymatic hydrolysis of cellulose has been ongoing for nearly 50 years. In 1950, E. T. Reese and his associates suggested that the enzymatic hydrolysis of natural cellulose involves two systems that act consecutively. The original model postulated that the C_1 system acts first to make the substrate more accessible to hydrolytic conversion by the C_x system [16]. As protein purification techniques improved and the enzymes composing a cellulolytic system could be isolated, the current hypothesis of three enzyme groups (cellobiohydrolases, endoglucanases, and β-glucosidase) working in concert was proposed. This hypothesis is based on a series of reactions outlined in Table 2.

Because cellobiohydrolases and endoglucanases act on insoluble cellulose, the rate of enzyme adsorption correlates to the rate of enzyme-substrate complex formation. Since β-glucosidase acts upon soluble cellobiose, Michaelis-Menten kinetics can be used to model its activity. This is an example of the uti-

Table 2. Cellulolytic enzyme reactions

Enzyme	Reaction Sequence[a]
Cellobiohydrolase	$E_1+G_x \leftrightarrow E_1G_x \rightarrow E_1G_2G_x \rightarrow E_1 + G_2 + G_x$
Inhibition	$E_1+G_2 \leftrightarrow E_1G_2$
Inhibition	$E_1+G \leftrightarrow E_1G$
Endoglucanase	$E_x+G_x \leftrightarrow E_xG_x \rightarrow E_x2G_x \rightarrow E_x+2G_x$
Inhibition	$E_x+G \leftrightarrow E_xG$
β-Glucosidase	$E+G_2 \leftrightarrow EG_2 \rightarrow E2G \rightarrow E+2G$
Non-competitive Inhibition	$EG_2+G \leftrightarrow EG_2G$
Competitive Inhibition	$E+G \leftrightarrow EG$

[a] E_1 = cellobiohydrolase; E_x = endoglucanase; E = β-glucosidase; G_x = cellulose chain; G_2 = cellobiose; G = glucose.

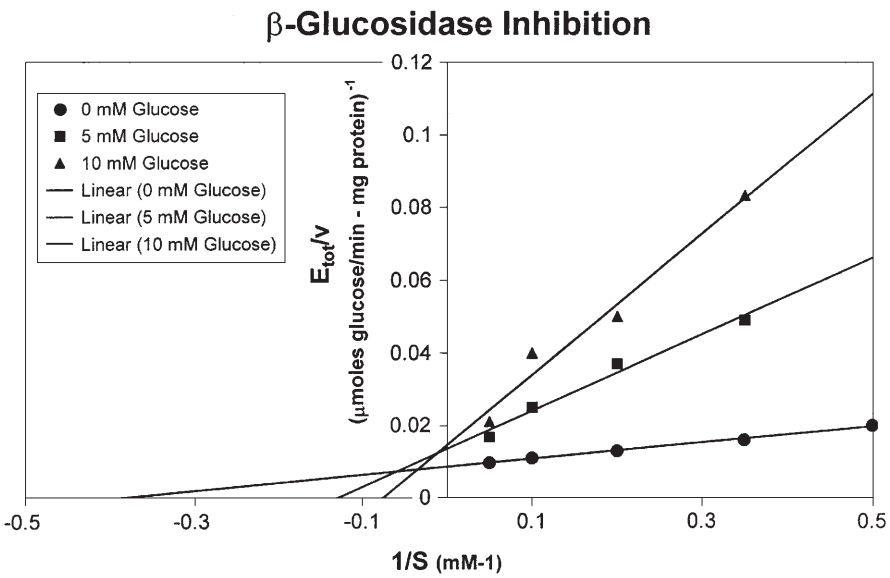

Fig. 3. β-Glucosidase inhibition shown by Lineweaver-Burk plot (reproduced from [2]). Lineweaver-Burk plot of kinetic data from "peak 2 cellobiase" (β-glucosidase) at several product inhibitor levels. This is an example of noncompetitive inhibition where the product is not only completing for binding in the active site but also binding to a secondary site on the enzyme that alters the enzyme catalytic ability

lity of operational kinetics. For example (Fig. 3), the initial rate kinetics of β-glucosidase showed initial rate patterns characteristic of non-competitive product inhibition [2]. Another enzyme characteristic, substrate inhibition, was shown in other experiments where enzyme velocity increased with increasing substrate concentration through a maximum. A slow decline in enzyme velocity for the conversion of cellobiose to glucose was noted as the substrate concentration was increased from the maximum velocity value [17]. In addition, cellobiohydrolase and endoglucanase have been shown to be inhibited by their hydrolysis products. Both are inhibited by cellobiose and glucose [5]. One theory is that the inhibition converts the normal enzyme substrate complex to one that is ineffective. Ratios of ineffective bound enzyme to total bound enzyme show an exponential decrease with increased cellobiose concentration and a linear decrease with increased glucose concentration.

One important point to note is that endoglucanases do not directly contribute to the generation of soluble saccharides from insoluble cellulose. Many studies on the kinetics of purified endoglucanases have shown glucose, cellobiose, and cellotriose are produced from small, soluble β 1–4 glucose oligomers. Although endoglucanases hydrolyze β 1–4 glycosidic bonds, they yield insignificant amounts of soluble saccharides from insoluble cellulose. Instead, the primary function of endoglucanases is to increase the number of free insoluble cellulose chain ends. Unfortunately, the number of free chain ends is a

parameter that is difficult to measure with any accuracy and must be considered for models based solely on these equations.

Because free cellulose chain ends are so difficult to quantify, other measurable cellulose characteristics have been shown to be factors correlating to hydrolysis susceptibility and rate [15, 18]. These factors affect how well the cellulose-binding domain, CBD, adheres to the cellulose and how well the catalytic domain acts on the cellulose. Because of what can and cannot be measured easily, models of enzymatic cellulose hydrolysis combine adsorption behavior with kinetic action.

6 Adsorption Modeling

Many adsorption models have been used for modeling cellulolytic enzyme adsorption, including Langmuir [15], two site Langmuir [19], and combined Langmuir-Freundlich models [20]. One of the earlier models utilized the unpurified *T. reesei* cellulase system as the basis for a Langmuir adsorption-hydrolysis model on Solka Floc [15, 18]. By using unpurified cellulase, many problems are inherent in this model. *Trichoderma reesei* cellulase system consists of seven different enzymes with affinities for adsorbing to cellulose varying from negligible (β-glucosidase) to very strong (cellobiohydrolase I) [21]. Because total protein was measured in these experiments, impurities of other proteins also influence the data.

Endoglucanase activity can increase pores and hydrolysis itself by reducing particle size and changing specific surface area. Changes in crystallinity index due to cellulolytic enzyme activity must also be taken into account. Effect models for these phenomena have not been generated. Another study demonstrated these effects in long-term rate kinetics of hydrolysis again using Solka Floc [18]. It was concluded that the two main factors in the decrease of long-term rates were the change in cellulose structure and the inhibitory effects of products. It was initially found that the specific surface area increased but then leveled off at 24 h (Fig. 4).

Increased specific surface area would thus increase the rate of hydrolysis. The increase in specific surface area is likely due to endoglucanase action of fragmenting the cellulose and opening pores thus increasing the amount of enzymes adsorbed. The crystallinity index, on the other hand, increased greatly in the first 12 h but then slowly decreased but not to its initial level. The crystallinity index increased due to the fast removal of the amorphous cellulose in relation to the crystalline cellulose.

Hydrolysis is the rate-limiting step. The form that the cellulose assumes also greatly affects hydrolysis rate. Initial kinetic rates for cellulolytic enzymes on soluble cellulose (soluble oligosaccharides and carboxymethyl cellulose) are many times greater than on insoluble cellulose [22]. Pretreatment of cellulosic materials is very important for increasing hydrolysis rates as well as overall conversion percentages [23]. Also, the action of the endoglucanase becomes increasingly important by increasing the number of free cellulose chain ends for continued cellobiohydrolase hydrolysis.

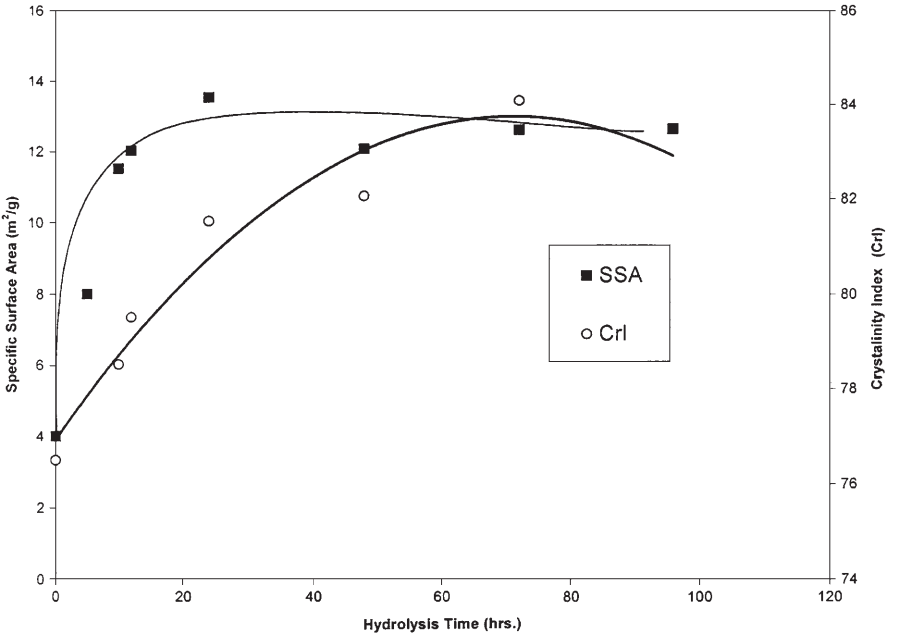

Fig. 4. Changes in specific surface area and crystallinity index by enzymatic hydrolysis (reproduced from [18]). Specific surface area (SSA) and crystallinity index (CrI) change quickly as enzymatic hydrolysis proceeds until leveling out at a constant level. Initial enzymatic action breaks cellulose into smaller particles (increased SSA) and attacks amorphous regions (increased CrI)

Work needs to be done to find the specific activity of purified enzymes on the cellulose structure, especially the synergism between different cellulolytic enzymes. With this understood, one can then undertake the task of studying the endoglucanase-cellobiohydrolase system. This requires the identification of binding sites, reaction sites, and changes in cellulose structure as a result of the enzyme action.

7 Cellulolytic Systems and Synergism

Cellulolytic systems require representation from all three groups of cellulolytic enzymes. As the currently accepted three-enzyme group hypothesis implies, these enzymes work not only in concert to hydrolyze cellulose, but also synergistically. Three types of synergism have been identified. The first and most obvious is endo-exo synergism. Endogluconases (endo), by random scission, generate more chain ends for cellobiohydrolases (exo) to attack. The effects of the other two types of synergism, exo-exo and intramolecular synergism are more subtle and have been discovered through careful research with pure cellulolytic enzymes [20].

Exo-exo synergism refers to the effects of two exo acting enzymes acting in concert compared to their individual activities. In the *T. reesei* cellulase system, for example, cellobiohydrolase I and cellobiohydrolase II act synergistically. Cellobiohydrolase I attacks the reducing end of the cellulose chain while cellobiohydrolase II attacks the non-reducing end of the cellulose chain [24–27].

Intramolecular synergism refers to the relative activites of adsorbed or non-adsorbed enzyme and the extent of adsoption of the catalytic and celllulose binding domains separately. Limited proteolysis studies have discovered that most cellulolytic enzymes have independent modules that have different functions. The catalytic domain has a lower rate of hydrolysis when separated from the cellulose binding domain [16]. The adsorption of the complete cellulolytic enzyme is also synergistically higher than the adsorption of either the cellulose binding domain or the catalytic domain alone [28]. This intramolecular synergism is tied closely to enzyme structure. Molecular architecture refers to the tertiary structure of enzymes and the role enzyme structure plays in creating enzyme activity.

8 Molecular Architecture

The majority of cellulolytic enzymes, cellobiohydrolases, and endoglucanases are modular proteins with two distinct, independent domains [29]. The first domain is responsible for the hydrolysis of the cellulose chain. The second is a cellulose binding domain, CBD, that has the dual activity of increasing adsorption of cellulolytic enzymes onto insoluble cellulose and affecting cellulose structure. By intercalating between fibrils and surface irregularities of the cellulose surface, cellulose binding domains help to reduce particle size and increase specific surface area. Microscopy of cellulose treated with isolated cellulose binding domains generated from recombinant organisms has shown the release of small particles from insoluble cellulose with no detectable hydrolytic activity and an increase in the roughness of highly crystalline (70–74%) ramie fibers [30].

Overall, cellobiohydrolases are one of the most important cellulolytic enzyme groups, because cellobiohydrolase I (CBH I) makes up 60% of the protein mass of the cellulolytic system of *Trichoderma reesei* [31], and its removal by gene deletion reduces overall cellulase system activity on crystalline cellulose by 70% [26]. Early work that discovered the modular nature of most cellulolytic enzymes focused on cellobiodydrolase I from *T. reesei*. Limited proteolysis studies completed more than a decade ago showed that cellobiohydrolase I contains two functional domains [32]. The C-terminal glycopeptide (10 kDa) acts as a binding domain for insoluble cellulose, whereas the core protein (55 kDa) contains the hydrolytic active site. X-ray scattering studies have shown that cellobiohydrolases [31, 33] and endoglucanases [6] are "tadpole" shaped with the catalytic core forming the "head" and the wedge-shaped cellulose binding domain at the tip of the "tail" (Fig. 5). The major part of the "tail" is composed of a flexible, heavily *O*-glycosylated linker region, approximately 30–44 amino acids in length, and rich in proline, glycine, serine, and threonine [34].

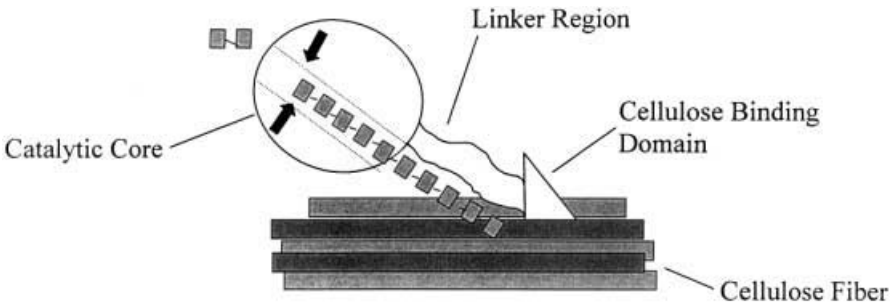

Fig. 5. Modular nature of cellulolytic proteins. The enzyme illustrated (cellobiohydrolase) binds to the cellulose microfibril through the cellulose binding domain while the catalytic domain binds a single cellulose chain at a free end. *Arrows* representing the active site within the catalytic domain show the ability of cellobiohydrolase to remain bound to the substrate while the hydrolyzed product, cellobiose, is released. This allows the enzyme to progressively hydrolyze cellobiose from cellulose

8.1 Catalytic Core

More recently, high resolution electron density mapping of the cellobiohydrolase I [26] and cellobiohydrolase II [33] has elucidated the nature of the catalytic domains. From this information, a hypothesis has been generated to explain the activity of these enzymes. In cellobiohydrolase I, two large anti-parallel β sheets that stack face-to-face occupy about one-third of this 434-residue domain. These two highly curved β sheets form a flattened cylindrical tunnel approximately 40 Å long that accommodates the cellulose chain with seven proposed glycosyl binding sites of similar aromatic residue structure. Although the catalytic core of cellobiohydrolase II, also from *T. reesei*, does not have this β sandwich, a similar tunnel structure is formed by several long α helices with four similar glycosyl binding sites [33]. In both cellobiohydrolase I and cellobiohydrolase II, two acidic residues lie near the second glycosyl bond of a bound cellulose chain. One lies above and one lies below the glycosyl bond. One residue acts as a proton donor while the other acts as a nucleophile [35]. The cleavage of this bond frees cellobiose, which can then leave the end of the tunnel. This molecular architecture allows these enzymes to release the product while remaining bound to the cellulose chain. The enzyme can then progress along the cellulose chain releasing cellobiose. One major difference between the two *T. reesei* cellobiohydrolases is that cellobiohydrolase I proceeds toward the non-reducing end and cellobiohydrolase II proceeds toward the reducing end of the cellulose chain [36].

The tunnel active site also explains why only cellobiose, not glucose or cellotriose or any other oligosaccharide, is produced. The cellulose polymer is ribbon-like with the glycosyl bonds alternating sides. Limited tunnel volume prevents extensive cellulose chain rearrangement that would allow more variation in the products generated (Fig. 6).

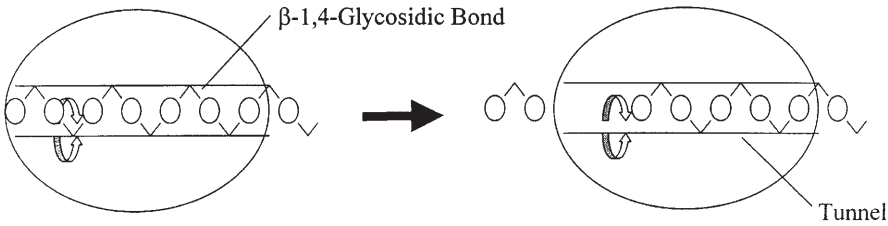

Fig. 6. Cellobiohydrolase-cellulose interaction. In the cellulose chain, the β 1–4 glycosidic bond alternate sides of the ribbon-like structure. Steric hindrance prevents the cellulose chain from bending or turning within the catalytic domain of cellobiohydrolases. This forces the product into dimer units as the enzyme progresses along the chain

Endoglucanases have similar structure and functionality to cellobiohydrolase, although a larger variety of hydrolysis products are produced. Early hypotheses suggested that endoglucanase was attacked by random scission of amorphous cellulose while cellobiohydrolase attacked chain ends. Recent X-ray diffraction studies of endoglucanase I from *Trichoderma reesei* have produced data that further confirm this hypothesis [6]. The overall molecular architecture of endoglucanase I is very similar to cellobiohydrolase I and cellobiohydrolase II. The major difference lies in the catalytic domain. The tunnel-forming loops found in cellobiohydrolase I are missing in endoglucanase I, which results in an open clef active site. Because there is less restriction in the binding of cellulose in the endoglucanase active site, many different hydrolysis products can be formed, including glucose, cellobiose, and cellotriose from soluble cellulose oligomers. This difference in specificity of product formation is one characteristic that separates cellobiohydrolases and endoglucanases into distinct classes of cellulolytic enzymes.

8.2 Cellulose Binding Domain

The cellulose binding domains (CBDs) of cellulases are highly conserved between all cellulases, especially within the species of a cellulase source. Cellulose binding domains have been grouped into three families based on sequence homology, which are defined independently of the sequence homology families of glycosyl hydrolases discussed above. Family I cellulose binding domains are found in fungi like *T. Reesei*, and families II and III are bacterial cellulose binding domains.

Family I cellulose binding domains consists of around 35 amino acids that are highly conserved between the two types of cellobiohydrolases and the four types of endoglucanases of *T. reesei*. The amino acids most likely responsible for binding are three aromatic residues, two tyrosines, and one tryptophan, and a combination of two polar residues (proline, glutamine, and asparagine). These groups are arranged on two β-sheets so that they allow the aromatic residues to bind to the face of the sugars and the polar residues to lie above the interglycosal bonds and hydroxyl groups of the cellulose chains. Point mutagenesis of *T.*

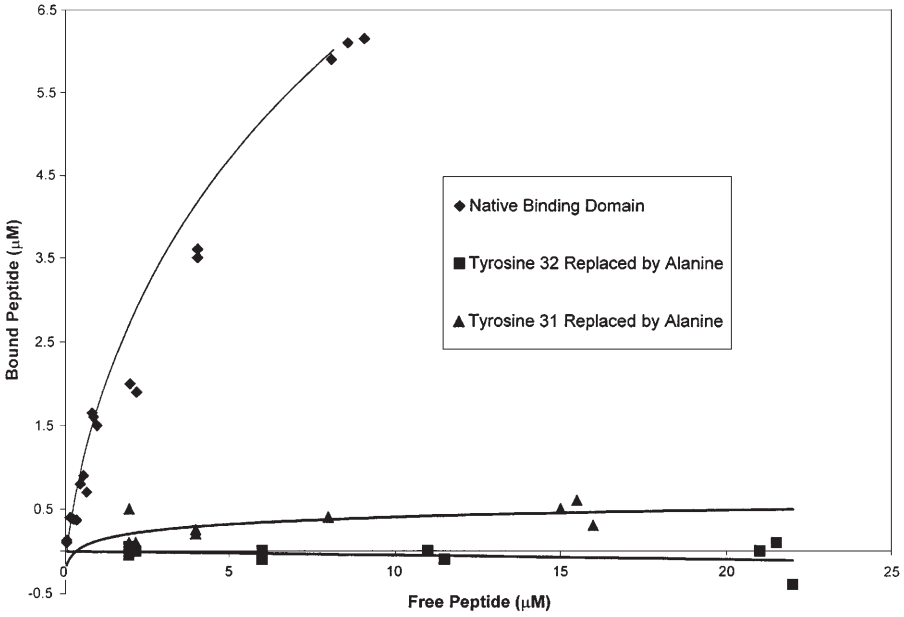

Fig. 7. Adsorption isotherms for mutated cellulose binding domains (reproduced from [13]). This graph illustrates the importance of tyrosine residues for cellulose binding in the cellulose binding domain. NMR comparisons between the native binding domain and the mutated domains were given in [13]. Note that Y32 A, which shows no affinity for crystalline cellulose, showed very slight chemical shifts in structure from the wild type as measured by NMR

reesei cellobiohydrolase I DNA sequence has shown that these aromatic residues are likely important for binding [13]. Replacing the tyrosines on this flat face with alanine showed a marked decrease in adsorption to crystalline cellulose (Fig. 7).

The family II and family III cellulases consist of a similar structure that is much larger than family I cellulose binding domains (100–170 amino acid residues) with three aromatic amino acid residues, mainly tryptophan, and five to seven polar residues. Again these amino acids bind to the cellulose with the aromatic residues associated with the sugar face and the polar residues with the hydroxyl groups. Families II and III cellulose binding domains also require a calcium ion to stabilize the binding domain and to function properly [12].

8.3 Linker Region

Joining the independent catalytic core to the cellulose binding domain is a linker region. This region varies between 6 and 59 amino acid residues in length depending upon the source of the enzyme. Although very little apparent sequence homology between different enzymes has been noted, all of these sequences are rich in proline and hydroxyl amino acids [29]. Although little is known about

the true importance of this structure, it is hypothesized that it effectively separates the catalytic core from the cellulose binding domain to allow them to function independently. Genetic mutation of the gene causing the shortening of the linker region was shown to impare binding and hydrolysis rates [34]. In fungal cellulolytic enzymes, this linker region also has several *O*-glycosylation sites. Although limited studies have been conducted on the importance of the linker region, the effect of the *O*-glycosylation has not yet been discovered [34].

9 Chemical Mechanism

Cellulolytic enzymes, like all glycosyl hydrolases, hydrolyze glycosidic bonds via the mechanism of general acid catalysis. Two critical amino acid residues, a proton donor and a nucleophile/base, are required for general acid catalysis. In most cases, the two amino acid residues implicated for these roles are glutamic and aspartic acids. Besides proximity of the appropriate donor proton and nucleophile, the energy barrier is also lowered by substrate distortion due to binding and stabilization of the chemical intermediate of the reaction. Cellulolytic enzymes utilize all of these factors in catalyzing the hydrolysis of the cellulose chain [35].

Hydrolysis, as the name suggests, is the breaking of bonds by adding water. In glycosyl hydrolases, this is accomplished using two different means. The first uses the charged environment of the catalytic site to "activate" a water molecule to act as a nucleophile while an acidic amino acid residue donates the required proton. This is called the inverting mechanism (Fig. 8) because the protonation of the catalytic residues alternates or inverts at the completion of the hydrolysis reaction, i.e., the charged moiety becomes protonated as the result of the nucleophilic attack of the activated water while the proton donor becomes the charged moiety that activates the water molecule for the next reaction.

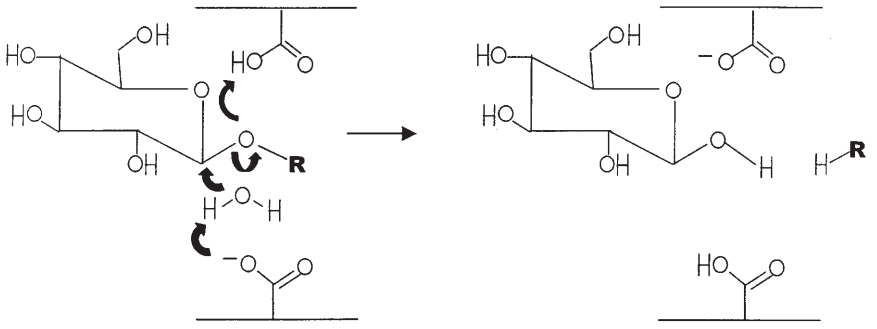

Fig. 8. Glycosyl hydrolysis – inverting mechanism. The inverting mechanism of glycosyl hydrolysis utilizes a protonated acidic amino acid residue as a proton donor. A charged acidic amino acid residue opposite the proton donor "activates" water for nucleophilic attack by electrostatic repulsion. The remaining proton from the water protonates the residue to reset the active site with the roles now reversed

The second mechanism, called the retaining mechanism, forms a covalently bound intermediate through nucleophilic attack of the charged amino acid on the glycosyl bond. A second step, where a water molecule is activated for nucleophilic attack, frees the hydrolysis product from the enzyme and recharges the proton donor (Fig. 9).

The major difference between inverting and retaining glycosidases is the distance between the two acidic residues. For ten structurally defined glycosidases, the average distance between the catalytic residues (for the four possible distances between carboxyl oxygens) is 4.8–5.3 Å for the retaining glycosidases while the distance is 9.0–9.5 Å for the inverting enzymes. The significantly greater separation is presumably necessary in inverting enzymes where both water and the substrate must be positioned between the carboxyl groups [35].

Cellulolytic enzymes of both types, inverting and retaining have been discovered [36]. Cellobiohydrolase I, the most abundant enzyme in the *T. reesei* cellulase system, is a retaining enzyme. Two glutamic acid residues have been suggested as likely candidates required for catalytic activity. One lies below and one lies above the β(1–4) linkage that is hydrolyzed [26]. These effective catalytic structures, coupled with the structural architecture that facilitates protein binding and progressive hydrolysis along the cellulose chain, make these enzymes efficient in the hydrolysis of cellulose.

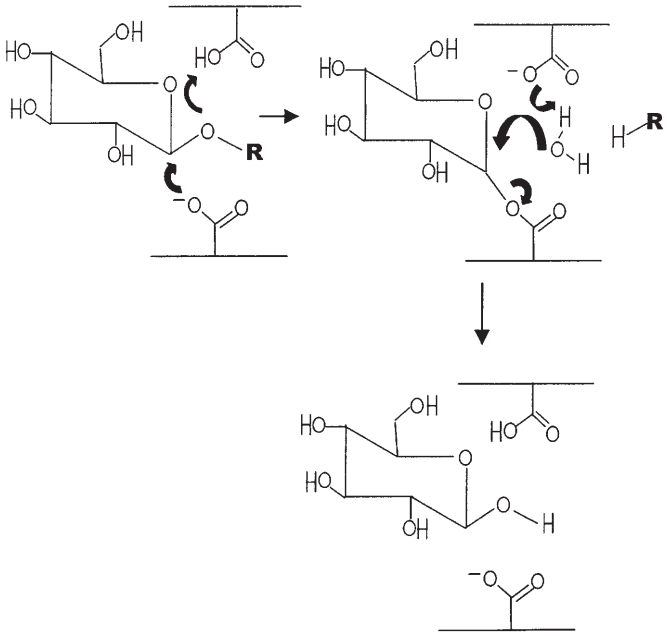

Fig. 9. Glycosyl hydrolysis – retaining mechanism. The charged acidic acid residue in the active site of a retaining glycoside hydrolase nucleophilically attacks the glycosidic bond while an opposing protonated residue donates the necessary proton. The covalently bound intermediate product is released by a second nucleophilic attack by a water molecule "activated" by electrostatic repulsion from the recently de-protonated residue

10 Conclusions

Fundamental studies of the molecular basis of cellulolytic enzymes show surface interactions are a key determinant of enzyme efficiency. Retaining and inverting mechanisms have recently been shown to have similar operations in cellulose hydrolysis as in the general-acid catalytic mechanisms of other glycolsyl hydrolases. Specific physical and chemical characteristics of the solid substrate have also been shown to influence greatly the hydrolysis rate of cellulolytic enzymes.

The elucidation of the molecular action of cellulolytic proteins has increased the potential applications of these enzymes to many different areas. Understanding of cellulolytic enzyme kinetics and mode of action has revolutionized the detergent and textile industries. Cellulolytic enzymes are currently being used as means of finishing cellulose fabrics such as rayon, linen, and cotton. Using the knowledge of the mode of action of endoglucanases and cellobiohydrolases, products for industrial applications that have controlled and predictable activities have been developed. These products are being used to "stone wash" denim, and "biofinish" other textiles to prevent fuzzing and pilling, increase smoothness and softness, and increase color brightness [1]. Further studies of the synergistic interactions of different cellulolytic enzymes will lead to the production of more efficient cellulase systems for use in the saccharification of biomass [37].

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Cellulase from Submerged Fermentation

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Commercial production of cellulase enzymes by submerged culture fermentation began in the early 1970s, with cellulase made by *Trichoderma* sold for use in research and pilot studies. The mid-1980s saw the first large industrial uses of cellulase for stonewashing denim and as an additive for animal feeds. This was accompanied by the introduction of commercial cellulases made by fungi of the genera *Aspergillus, Penicillium*, and most importantly *Humicola*. By this time, the productivity of cellulase in commercial fermentations was over 400 IU l^{-1} h^{-1}. Growth in cellulase use has continued into the late-1990s with other textile applications such as biopolishing, animal feed applications in increased digestibility of barley and wheat-based feeds, clarification and yield improvement for fruit juice, and in household laundry detergent.

Keywords. Cellulase, Fermentation, Submerged, Culture

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1 Introduction

During World War II, the US Army was concerned about the deterioration of cotton tents and clothing in the South Pacific. The Army set up several efforts to find immediate solutions to the problems and undertook a long term basic research program within the Quartermaster Corps to investigate the nature of the rotting, including the causal organisms, the mechanism of action, and methods of control. Toward this end, an eight-person research group was established under the leadership of Elwyn T. Reese at the US Army laboratories at Natick, Massachusetts [1, 2].

Reese and coworkers found that the microorganisms responsible for the cotton deterioration as being able to secrete extracellular enzymes capable of degrading cellulose. By 1953, Reese determined that natural cellulase enzymes are complexes of several different enzymes with different abilities to degrade cellulose. Reese speculated that there were three types of enzyme activities involved in hydrolyzing cellulose: C1, which converts crystalline cellulose to amorphous; Cx, which hydrolyzes amorphous cellulose to cellobiose; and beta-glucosidase, which hydrolyzes the soluble dimer cellobiose to glucose. He had further identified fungi of the genus *Trichoderma* that produce relatively large amounts of cellulase that are particularly potent in hydrolyzing cellulose to glucose. One of Reeses strains was named *Trichoderma reesei* in his honor in 1977 [3].

Reese was joined at Natick in 1956 by Mary Mandels, and the focus of cellulase research changed from preventing cellulose hydrolysis to enhancing it, such as for replacing starch in the production of glucose. Mandels and Reese determined that cellulase production by *Trichoderma* in liquid culture was induced by sugars such as cellobiose and, more potently, sophorose, and determined bet-

ter media formulations and growth conditions. Mandels and Reese also selected for *Trichoderma* mutants with higher productivity of cellulase production, reaching a level of 3 International units per liter per hour (IU $l^{-1} h^{-1}$) for the first time [4, 5].

The research interest in cellulase increased dramatically in the 1970s, as the use of cellulase to produce sugar from cellulose that could be fermented to ethanol by yeast was considered as an alternative fuel during the gasoline shortages. Dozens of cellulase-producing strains were identified and improved. An improvement in cellulase productivity in *Trichoderma* to over 100 IU $L^{-1} h^{-1}$ was achieved using selection procedures developed by Bland Montenecourt of Lehigh University [6, 7]. Reese's speculation about the types of activity carried out by cellulase enzymes was refined to the point of identifying cellobiohydrolases (CBH), which act along the glucose polymer releasing the soluble dimer cellobiose, and endoglucanases (EG), which act at random locations on the glucose polymer.

The modern techniques of molecular biology were brought to bear in cellulase research in the 1980s. Cloning and expressing a cellulase gene in a foreign microbe were successful in 1982 and 1983 [8, 9]. The existence of distinct catalytic and binding domains in cellulase enzymes was identified in 1986 [10]. By the late 1980s, chemical modification and protein engineering were used to study specific elements of cellulase enzymes. In the 1990s, protein engineering was used to alter the properties of the enzymes [11, 12]. The three dimensional structure of *Trichoderma* CBHII was reported in 1990 [13] and of many other cellulase enzymes thereafter.

Commercial production of cellulase enzymes by submerged culture fermentation began in the early 1970s, with cellulase made by *Trichoderma* sold for use in research and pilot studies. The mid-1980s saw the first large industrial uses of cellulase for stonewashing denim and as an additive for animal feeds. This was accompanied by the introduction of commercial cellulases made by fungi of the genera *Aspergillus, Penicillium*, and most importantly *Humicola,* introduced by Novo in 1986 [14]. By this time, the productivity of cellulase in commercial fermentations was over 400 IU $L^{-1} h^{-1}$ [15]. Growth in cellulase use has continued into the late-1990s with other textile applications such as biopolishing, animal feed applications in increased digestibility of barley and wheat-based feeds, clarification and yield improvement for fruit juice, and in household laundry detergent.

The volume of cellulase produced by submerged fermentation is much larger than that produced by solid culture, which is primarily limited to baking and Asian detergent applications. Worldwide consumption of cellulase from submerged fermentation is roughly 23,000 tonnes annually. The sales volume of cellulase is around $125 million, which represents over 10% of all industrial enzyme sales.

2
Applications of Cellulase Enzymes

2.1
Stonewashing Denim

Denim stonewashing originated in the 1970s as a way to deliver pre-softened blue jeans to the public. The sewn denim was washed in the presence of pumice stones for roughly 60 minutes to shear and abrade the garments. The resulting jeans were softened by the stonewashing and therefore "ready to wear" at the time of purchase. This was preferred by the consumer over the previously-available stiff jeans that required an extensive "wear-in" period before they were comfortable. In addition, fashion favored faded jeans over the traditional dark blue jeans, and stonewashing removed a portion of the indigo dye, creating a faded appearance.

Although the marketplace favored stonewashed jeans, the use of stones caused several problems to denim washers. The stones damaged the washing machines, provided dust in the plant and the process effluent, and the handling of stones caused numerous worker injuries.

In the late 1980s, the use of cellulase enzymes began as an alternative to stones. Cellulase gained a foothold in the industry by producing the softness and appearance of jeans washed with pumice stones, but without the problems of stones. The availability of cellulase at costs competitive with stones in early 1990 led to the widespread adoption of cellulase use by the industry. Today, cellulase is used all over the world in this application, sometimes alone and sometimes with some stones present.

The original cellulases used in denim washing were the crude enzymes of *Trichoderma* and *Humicola*, referred to as "acid" and "neutral" cellulase, respectively, based on the optimum pH range of use of the enzymes, which was pH 4 to 5 for the acid cellulase and 6 to 7 for the neutral cellulase. The *Trichoderma* cellulase, comprising the more complete set of EG and CBH components capable of the full hydrolysis of cellulose, works more quickly and is capable of a greater degree of abrasion and fading of the blue dye color than the *Humicola* cellulase. The *Trichoderma* cellulase also achieves certain desired "finishes" (appearances) that the *Humicola* cellulase does not.

The *Humicola* cellulase works on the garments more gently, resulting in a higher retention of fabric strength. In addition, the *Humicola* cellulase results in less backstaining of the indigo dye onto the white threads and pockets of the denim. Finally, the neutral pH range of the *Humicola* cellulase facilitates easier and more forgiving pH adjustment of the wash, which is naturally slightly alkaline. For much of the market, the balance of advantages lay with the *Humicola* cellulase, which fostered a premium product status for it. However, the *Trichoderma* cellulase was still widely used.

By the mid-1990s, several new cellulase enzyme products had evolved for denim washing. Lower degrees of fabric strength loss were achieved with individual cellulase enzyme components, most prominently *Trichoderma* EG3 [16] and *Humicola* EG5 [17]. *Trichoderma* cellulase used in combination with protease improved its performance to close to that of the *Humicola* cellulase [18].

Some specialized cellulases are used to minimize dye streaking on the fabric, to cause more abrasion near seams, or any of a myriad of other desired effects. Cellulases operating at high pH (> 9) have not been successful, as alkaline pH is used to terminate the enzyme reaction to prevent unwanted damage, and this procedure is ineffective with alkaline cellulase.

In terms of enzyme product formulations, liquid and granulated enzymes are used. Cellulase is combined with surfactants to give a cleaner finish.

2.2 Household Laundry Detergent

The use of cellulase in household laundry detergent originated in 1993 with the introduction of *Humicola* EG5 into "New Cheer with Advanced Color Guard". Cellulase in laundry detergent removes the hairs, known as pills, that occur on cotton clothes after repeated wearing and machine washing. The cellulase removes the existing pills, and conditions the surface of new or unpilled clothes. The result is an appearance that more closely resembles a new garment in sharpness of color and smoothness of appearance. Cellulase also enhances the softness and removal of soil from the garment. The use of cellulase can eliminate the need for cationic fabric softeners, which have disposal and cost problems [19].

Household laundering is carried out at pH 8 to 9.5, and it is essential that the cellulase be active in this pH range. For this reason, the *Trichoderma* cellulases have not been successful in this application. The enzyme must also withstand potential inhibitors and inactivators, among them protease, surfactants, and bleach. The EG5 has been modified by protein engineering to improve its performance in detergent systems by increasing its tolerance of anionic surfactants, protease, and peroxide bleach and increasing its ability to adsorb to cellulose [20]. Other cellulases used in household detergents include alkaline cellulase made by *Bacillus*, some of which is made in solid culture.

2.3 Animal Feed

Beta-glucanase enzymes were introduced to enhance the digestibility of animal feeds with limited success in the early 1970s. More recently, the cost-effective cellulase enzymes developed for other industries have been adapted for use in the feed industry.

The primary use of cellulase in the feed industry has been in barley- and wheat-based feeds for broiler chickens and pigs. The barley and wheat contain soluble beta-glucans that increase the viscosity of the feed in the gut of the animal. This, in turn, causes an uptake of water, which decreases the amount of carbohydrate and vitamins that the animal obtains from the feed, as well as causing sticky stool and related problems of disease and effluent disposal [21, 22]. Inclusion of cellulase in the feed, as well as xylanase and other enzymes, helps to overcome these problems.

This application is carried out with the crude cellulases of *Trichoderma, Aspergillus,* and *Penicillium*. The digestive process and action of cellulase in the

system is not well understood. It seems to occur at acidic pH and crude cellulases from all three of these microbes are used.

The most important parameter characterizing feed digestibility is the feed conversion ratio (FCR), which is the ratio of weight of feed consumed to weight gain by the animal. Commercial cellulase enzymes, at dosages of 100 to 1000 ppm in feed, decrease the FCR for barley and wheat feeds by 1% to 8% in field tests. This results in a significant cost savings to the farmers. The enzymes, which can be liquids or fine granules, are added directly to the feed just prior to consumption by the animals.

The use of cellulase in this application is most prominent in western Europe. The use of cellulase in corn/soy feeds, which are the most prominent in the US, has not been successful. Corn and soy are more easily digested than barley and wheat and less apt to result in a highly viscous feed.

Smaller applications of cellulase enzymes from submerged fermentation are in textile biopolishing, deinking and dewatering paper, processing of fruit juice and other beverages, baking, and alcohol production.

2.4 Textile Biopolishing

Biopolishing with cellulase is analogous to the use of cellulase in household laundry detergent. The enzyme removes pills from the fabric, restoring its appearance and conditioning it for resistance to further pilling [14].

In the present context, "biopolishing" is used to denote enzymatic depilling in an industrial context as carried out on unsewn fabric. There is a growing use of cellulase enzymes in the textiles industry.

Many fabrics, but particularly blends of cotton and non-cotton fibers, develop a degree of hairiness during sewing or weaving. This hairiness is undesirable from the customers point of view, as it detracts from the overall appearance of the garment and the crispness of the colors. Lyocell, a blend of cotton and wood fibers, is particularly prone to pill formation.

The preferred method of removing the pills is to treat the pilled fabric with cellulase enzymes. This is typically carried out by loading the fabric onto a cylindrical drum that is partly submerged in liquor containing cellulase. The drum rotates at 50–100 rpm, which provides shear to help dislodge pills from the fabric. Cellulase treatment is typically for 15–30 min at 40–50°C, pH 5.

Most of the dyes used on Lyocell are more stable at acidic than alkaline pH, so this application has primarily been carried out using acidic cellulase from *Trichoderma*. The cellulase used can be the entire crude cellulase or modified cellulases with gentler action, depending on the size of the pills and the robustness of the fabric.

2.5 Deinking and Dewatering Paper

Two emerging applications of cellulase in the pulp and paper industry are in ink removal and water removal [23].

Deinking is the process by which the ink is removed from paper to allow it to be recycled. Deinking is carried out on low cost paper such as newsprint as well as fine paper, but cellulase is playing a role only in fine paper deinking at this time.

The conventional deinking process is by flotation. Fine paper, such as photocopier paper, is pulped in water at 5% to 20% solids consistency. The pulp is pumped to a flotation deinking basin, in which air is blown up through a sparger located near the bottom. The ink particles migrate to the air/liquid interfaces and float to the top of the basin. Surfactants are added to promote the migration of the ink particles. The ink is skimmed off the surface and the deinked pulp is bleached with hydrogen peroxide and sodium hydrosulfite, or in some cases more aggressive bleaching chemicals like chlorine dioxide or ozone.

Cellulase is added during pulping or flotation deinking. The enzyme increases the amount of ink removed from the fibers, thereby increasing the cleanliness of the sheet. This results in a brighter, cleaner sheet, or alternatively a reduction in the use of surfactants and bleaching chemicals.

The mechanism of the enzymes action in deinking is not well understood. The desired pH range for treating alkaline paper, which has become much more common than acid paper, is pH 7 to 8 to minimize degradation of the calcium carbonate. This is suited to the *Humicola* and *Aspergillus* cellulase enzymes, which are used in this application.

Paper dewatering is most important on the paper machine, where an aqueous slurry of pulp and additives are pressed into paper sheets. The water must be removed from the sheets by a combination of pressing, vacuum, and heat. The economy of the paper machine improves the faster it forms dry sheets of paper. However, speeding up the paper machine decreases the length of time available for dewatering. Often, the speed of the paper machine is limited by the rate at which water can be removed from the sheets.

Cellulase enzymes increase the rate of drainage of pulp, thereby offering the potential to increase the speed of the paper machine. Cellulase solubilizes a portion of the small particles, called fines, which are highly water holding. The rate of drainage is quantified by the Canadian Standard Freeness (CSF) measurement. Cellulase treatment increases the CSF by 20 to 40 points.

Cellulase treatment is carried out in a slurry tank prior to the paper machine. The typical treatment time is for 1 hour. Primarily *Trichoderma* cellulase enzymes have been used for this application, which is carried out at pH 4 to 5.

2.6 Fruit Juice and Beverage Processing

In the production of fruit juice, wine, beer, and other beverages, the raw juice is in a slurry (known as a "mash") with solid fruit. The juice is separated from the fruit by a combination of filtration and/or centrifugation. The separation operation is a cost trade-off: the more powder, time, and wash water used, the higher the yield of juice, but the more costly the process. There are also trade-offs of clarity and yield.

Cellulase enzymes break down cellulose and beta-glucan associated with the cell walls, thereby decreasing the viscosity of the mash and increasing the ease of the juice recovery. The conversion of cellulose and beta-glucans into soluble

sugar provides another increase in the overall juice solids yield. The enzyme treatment can also increase the clarity of the juice by solubilizing small particles. The enzyme treatment can enhance the flavor of the juice by increasing the extractability of flavor compounds in the mash. Where disposal of the solid residue is costly, cellulase helps to decrease waste disposal costs.

Apple juice is the juice most commonly produced using cellulase enzymes, followed by cranberry juice, orange juice, and grape juice.

Although the benefits of cellulase are significant in juice production, pectinases are more important in these systems. Fruits contain high levels of pectin, and pectin is efficiently hydrolyzed by pectinases. Pectinase increases juice yields by 10% to 50%, while cellulase provides an enhancement of 5% to 15%. The effects of pectinase and cellulase together are additive and can even be synergistic.

Another concern in using cellulase in beverages, particularly beer and wine, is the possibility of changes in flavor. Although increasing flavor extractability is often desirable, many beer and wine brand names maintain a constant flavor that is undesirable to change.

Most cellulase used in the juice industry is *Trichoderma* cellulase, because of the typically low pH present in the mash. Cellulase from *Aspergillus niger* is also used.

2.7 Baking

Cellulase enzymes are used extensively in baking, but most of these are from solid culture. In general, the enzyme action desired in baking is very mild. Cellulase is used to break down gums in the dough structure, so as to allow a more even dough rise and flavor distribution. However, too much action can damage the dough structure and degrade the baked goods. *Trichoderma* cellulase, for example, is too aggressive in its hydrolytic action for cookie production, and its use in baking is restricted to crackers [24].

Among cellulases from submerged culture, *Aspergillus* is the most widely used in baking. *Aspergillus* cellulase is prominent where cellulase is used for baking breads and cakes. This stems from the use of *Aspergillus* cellulase from solid culture in baking.

2.8 Alcohol Production

Although not yet a commercial process, cellulase enzymes play a major role in the production of fuel alcohol from cellulose.

Ethanol from cellulose represents an enormous opportunity to make a transportation fuel that is an alternative to gasoline. Development of such a fuel is motivated by 1) an increased cleanliness of automobile exhaust, with decreased levels of carbon monoxide and nitrous oxides, 2) a need for a fuel that does not contribute to an increase in the Greenhouse effect, 3) the desire to decrease the dependence of the United States on imported petroleum, and 4) the possibility of creating wealth in regions where cellulose is a prevalent natural resource.

Cellulose is converted to ethanol by making glucose and then fermenting the glucose to ethanol using yeast. Cellulose conversion has been studied for many

years and has been carried out using 1) concentrated acid hydrolysis, 2) dilute acid hydrolysis, or 3) a combination of acid prehydrolysis and enzymatic hydrolysis. All three processes are under pilot-scale development. This discussion focuses on the acid prehydrolysis/enzymatic hydrolysis process.

For a cellulosic material such as wood chips or crop waste, cellulase enzymes cannot penetrate the structure and make glucose. A pretreatment is required to destroy the fiber structure and allow cellulase access to the substrate. Pretreatment is typically carried out at 180–250 °C for a few seconds to a few minutes in 0.5% to 2% sulfuric acid. The resulting material is of a muddy texture. An aqueous slurry of pretreated cellulose is made at 5% to 15% solids. Cellulase enzymes are added at a concentration of 5 to 25 filter paper units per gram of cellulose. The slurry is stirred, and the enzymatic hydrolysis is carried out for 4 to 7 days. At this point, most of the cellulose is converted to glucose, and the unhydrolyzed residue consists primarily of lignin.

The glucose syrup is removed from the residue by filtration and washing. The glucose is then fermented to ethanol, which is recovered by distillation.

The enzymatic hydrolysis process is carried out using crude *Trichoderma* cellulase at its optimum temperature and pH (50 °C, pH 5). Although the crude *Trichoderma* cellulase is highly efficient at hydrolyzing cellulose, at present it is not efficient enough for a commercial ethanol process to be viable. One problem is the shortage of beta-glucosidase, which causes cellobiose, a very potent inhibitor of cellulase, to accumulate. Beta-glucosidase is not only in short supply, but it is highly inhibited by glucose.

In an alternative process designed to address the accumulation of cellobiose, the enzymatic hydrolysis and ethanol fermentation are carried out simultaneously, using the so-called SSF (simultaneous saccharification and fermentation) process. SSF is designed to remove the glucose by yeast fermentation, thereby decreasing end-product inhibition of the beta-glucosidase by glucose, and allowing the beta-glucosidase to continue to hydrolyze the cellobiose. The disadvantage of SSF is that it must be carried out at a temperature that is compatible with the yeast (optimum 28 °C) and the cellulase (optimum 50 °C). The compromise temperature usually used, 37 °C, is suboptimal for both yeast and cellulase.

In addition to ethanol from cellulose, cellulase enzymes play a minor role in the production of ethanol from corn. In this process, most of the glucose is from starch. Cellulase enzymes offer the opportunity to increase the glucose yield by hydrolyzing a portion of the cellulose to glucose, as well as decreasing the viscosity of the ground corn [25].

3
Kinetics of Cellulase Enzymes

3.1
Substrate Specificity of Cellulase Enzymes

The activity of the *Trichoderma* cellulase enzymes CBHI, CBHII, EGI, and EGII against several substrates is summarized in Table 1.

Table 1. Activity of cellulase enzymes

Substrate	Cellulase component[a]				
	CBHI	CBHII	EGI	EGII	Reference
Beta-glucan	–	++++	+++++	+++	[26, 27]
Hydroxyethyl cellulose	–	+	++++	++	[26, 27]
Carboxymethyl cellulose	+	++	++++	+++++	[27, 29]
Methylumbelliferyl cellotrioside	–	–	–	+	[28]
Para-nitrophenyl glucoside	–	–	–	+	[27, 29]
Cellobiose	–	–	–	+	[27]
RBB xylan	–	–	+++	–	[26]
Crystalline cellulose	++++	+++	+	+	[29]
Amorphous cellulose	+	+++	+++++	+++	[29]

[a] Relative Activity: None: – Maximum: +++++.

Endoglucanases are the most active against soluble oligomers of glucose, such as beta-glucan or chemically-substituted cellulose, as well as amorphous cellulose. Cellobiohydrolases are most active against crystalline cellulose, with CBHI having little activity against other substrates. EGI is the least specific of the enzymes, as it has significant xylanase activity. None of the cellulase enzymes are especially active in solubilizing or hydrolyzing cellobiose.

3.2 Synergy Among Cellulase Enzymes

There is a significant level of synergy among cellulase enzymes, resulting from their different, but complementary, modes of action. Among the four major *Trichoderma* cellulase components, every pair is synergistic except EGI and EGII [29]. The synergy among the enzymes increases the degree of hydrolysis by more than two-fold over that achieved with individual enzymes.

3.3 Cellulase Assays

Although there are no assays of cellulase activity that are used universally, the most common assays are the Filter Paper (FPA) and carboxymethyl cellulose (CMCase) assays [30]. The filter paper assay indicates the ability of the enzyme to produce reducing sugars from cellulose filter paper. This assay is particularly appropriate for crude *Trichoderma* cellulase or other cellulases with high levels of CBH components. It is not as appropriate for endoglucanases. The CMCase assay measures the ability of the enzyme to produce reducing sugars from soluble CMC. This assay is particularly useful in measuring the activity of many endoglucanases, which have especially high activity against this substrate. A variant of the CMCase assay is to measure the drop in viscosity of a CMC solution caused by enzyme action. This is a particularly sensitive measure of the initial attack of CMC by an enzyme.

Regardless of the assay used, the non-linearity of cellulase kinetics requires that the enzyme activity be measured based on a fixed level of conversion.

3.4 Cellulose Hydrolysis

When faced with characterizing the kinetic behavior of an enzyme or a complex of enzymes, one usually pulls out a textbook on Michaelis-Menten kinetics and applies it to the system at hand. For beta-glucosidase, which hydrolyzes the soluble substrate cellobiose to glucose, this approach is fine. Unfortunately, for cellulase enzymes producing cellobiose from cellulose, this exercise is inadequate.

The fact that cellulase enzymes act on an insoluble substrate, cellulose, moves the kinetics outside Michaelis-Menten on several counts. First of all, the enzyme can be adsorbed to the substrate or unadsorbed, but only the adsorbed enzyme acts on the cellulose. Even more puzzling is the substrate concentration. Do we count the entire substrate, or just that in close contact with the enzyme? Clearly, we have to start from first principles in characterizing the cellulase/cellulose system.

3.5 Cellulase Adsorption

The first step in cellulose hydrolysis is the adsorption of the enzyme onto the cellulose. The rate of adsorption depends on the viscosity and agitation of the system. In a dilute, well mixed system, adsorption equilibrium is established in about 5 min. The adsorption equilibrium is described empirically by a Langmuir Adsorption isotherm [31]:

$$P_{\text{ads}} = \frac{K_{\text{p}} P_{\text{max}} P_{\text{L}}}{1 + K_{\text{p}} P_{\text{L}}} \tag{1}$$

where

P_{ads} adsorbed protein, mg g^{-1} cellulose
P_{max} saturation constant, mg g^{-1} cellulose
K_p binding constant, mL mg^{-1}
P_L protein in solution, mg mL^{-1}

For a natural substrate like ground cellulose or fruit pulp, P_{max} is of order 10 mg g^{-1}. Chemically pretreated cellulose such as paper pulp can have much higher P_{max} values. Higher dosages of cellulase than P_{max} have little effect on the rate of reaction. The adsorption behavior does not follow a reversible Langmuir profile, as the enzyme is very difficult to wash off its substrate. A Langmuir isotherm implies reversible adsorption.

3.6 Enzyme Action

Adsorbed enzyme acts on its substrate. Crude cellulase contains three types of enzymatic action against cellulose: CBHI, which acts along the glucose polymer

from the non-reducing end; CBHII, which acts along the glucose polymer from the reducing end; and endoglucanase, which acts randomly at points along the glucose polymer. There is a high degree of synergy between the action of these different cellulase enzymes.

The detailed kinetics of the individual cellulase components is a subject of research and will not be discussed here. However, some characterizations have been made by taking the cellulase complex as a whole. In this case, the behavior can be expressed as a function of the enzyme dosage by Eq. (2):

$$G = kE_{\text{ads}}^{\text{r}} \tag{2}$$

where
G glucose produced, mg g^{-1} cellulose
k rate constant
E_{ads} adsorbed enzyme concentration, mg g^{-1} cellulose
r reaction order

For cellulase, typically $0.15 < r < 0.7$. This fractional reaction order is characteristic of many effects by cellulase, not just production of glucose. The fractional reaction order indicates a diminishing return on increasing enzyme dosage. The relationship between extent of hydrolysis and reaction time is also expressed by a fractional exponent in time, which indicates a loss of enzyme effectiveness over time.

3.7
Product Inhibition, Enzyme Inactivation, and Substrate Recalcitrance

Cellulase is inhibited by its end products, cellobiose and glucose. For *Trichoderma* cellulase, the end product inhibition is uncompetitive, of the form:

$$\frac{r_{\text{i}}}{r} = \frac{1}{1 + \dfrac{I}{K_{\text{i}}}} \tag{3}$$

where
r = rate of hydrolysis, (g glucose) $L^{-1}\,h^{-1}$
r_i inhibited rate of hydrolysis, g $L^{-1}\,h^{-1}$
I inhibitor concentration, g L^{-1}
K_i inhibition constant, g L^{-1}

For crude *Trichoderma* cellulase, K_i for glucose is 69 g L^{-1} and K_i for cellobiose is 3.3 g L^{-1}.

Cellulase action can be inhibited or inactivated by several classes of compounds, including strong oxidants or reducing agents, metal ions, salts, solvents, and surfactants. The binding of the enzyme to cellulose can protect the enzyme from these compounds, so it is not easy to generalize the relationship between concentration and degree of inhibition (which is concentration dependent) or inactivation (which is time dependent).

For most cellulosic materials, there is an upper limit to the degree of conversion that can be obtained. At this point, the accessibility of the remaining cellulose to the enzyme is limited, either by its pore structure or the presence of noncellulosic components. The decrease in rate with increasing cellulose conversion is referred to as "substrate recalcitrance".

3.8 Effect of pH and Temperature

The fact that cellulase adsorbs onto its substrates and the action has a fractional reaction order has important effects on such practical issues as the pH and temperature ranges.

If one measures the enzyme activity as a function of pH or temperature using a soluble substrate, such as hydroxyethyl cellulose, one obtains curves characteristic of many enzymes. The temperature curve follows an Arrhenius dependence at temperatures leading up to the optimum, then drops sharply at inactivating temperatures (Fig. 1). The pH curve is roughly a bell shape, with the optimum spanning 1 to 3 pH units (Fig. 2). This exercise gives a first estimate of the pH and temperature curves, but often the behavior in specific applications is quite different.

Because the enzyme adsorbs to its substrate, the nature of the substrate influences the activity profiles. For example, as the pH is varied, the charge of the substrate and particularly ionic components of the substrate will change. This can effect the enzymes activity. For this reason, pH and temperature profiles for a given enzyme can vary widely among substrates.

The fractional reaction order dependence of the extent of reaction on enzyme dosage will tend to flatten the profiles observed in actual applications. For example, if an enzyme has a reaction order of 0.5 in a given application, then a

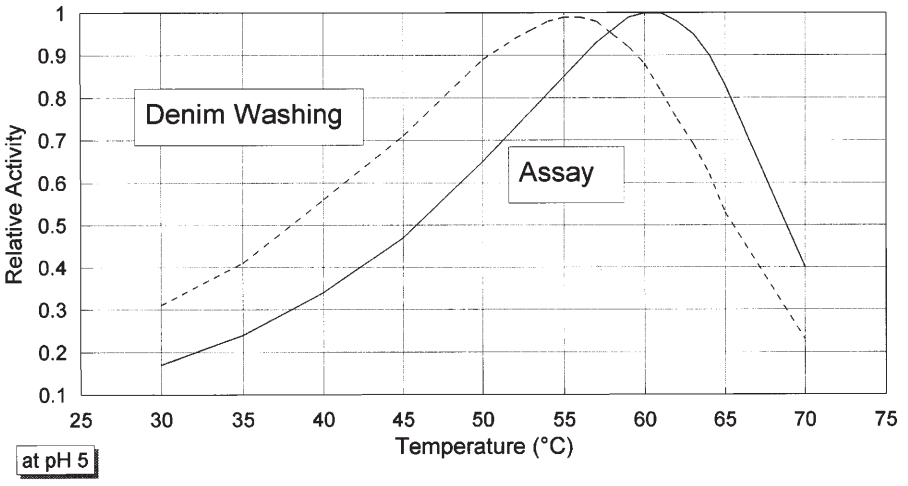

Fig. 1. Temperature profile of *Trichoderma cellulase*

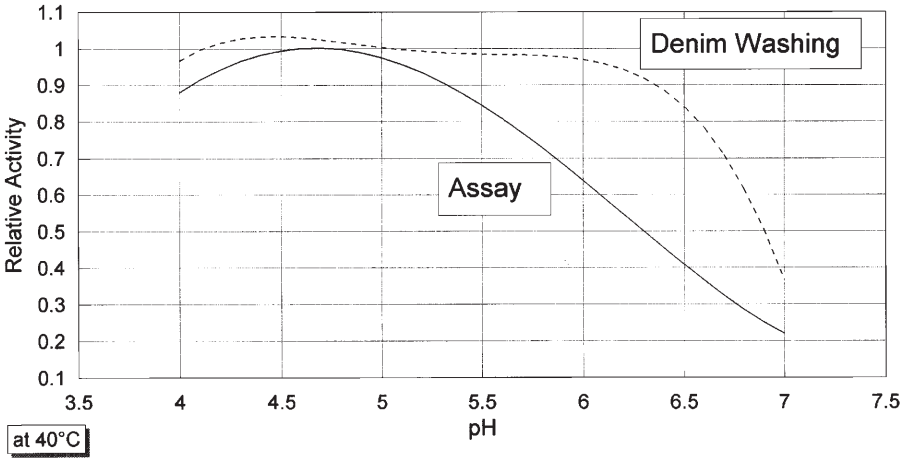

Fig. 2. pH profile of *Trichoderma* cellulase

4× change in enzyme activity (caused by a change in temperature) will only cause a 2× difference in the amount of enzyme action. Therefore, a graph of enzyme action as a function of temperature will have a flatter profile than a graph of enzyme activity as a function of temperature.

3.9
Effect of Shear

Although not easily described at the molecular level, the presence of shear in a system will act to increase the rate of cellulase action and the extent to which cellulase can modify a substrate. Shear can involve rubbing of the substrate against itself, such as denim in a washing machine, or abrading a substrate with a non-cellulosic material, such as pumice stones, or a combination of these. Shear acts to initiate fractures in the cellulose or propagate existing fractures. The net effect of shear is to increase the extent of reaction by the enzyme, or decrease the amount of cellulase required to reach a given effect.

The bound nature of the enzyme protects it from damage from any but the most intense shear.

3.10
Beta-Glucosidase Kinetics

Unlike the major cellulase components, beta-glucosidase is a soluble enzyme acting on a soluble substrate. Beta-glucosidase is characterized by classical Michaelis-Menten kinetics, with glucose acting as a competitive inhibitor [32]:

$$V = \frac{V_{\max} G_2}{G_2 + K_m \left(1 + \frac{G}{K_i}\right)} \tag{4}$$

Table 2. Beta-glucosidase Kinetic constants[a]

Constant	*Trichoderma longibrachiatum* BG	*Aspergillus niger* BG
V_{max} (g L^{-1} h^{-1}) @ 0.1 IU ml^{-1}	3.10	2.75
K_m (g L^{-1}')	0.16	0.36
K_i (g L^{-1}')	0.06	0.24

[a] 50 °C.

where
V reaction velocity, g L^{-1} h^{-1}
V_{max} maximum velocity, g L^{-1} h^{-1}
G_2 cellobiose concentration, g L^{-1}
K_m Michaelis constant, g L^{-1}
G glucose concentration, g L^{-1}
K_i inhibition constant, g L^{-1}

Values of the kinetic constants for beta-glucosidase from *Trichoderma* and *Aspergillus* are shown in Table 2.

Beta-glucosidase is available within the complex mixture of crude cellulase enzymes. In addition, some microbes, including *Aspergillus niger*, produce beta-glucosidase with little additional cellulase activity.

4 Production of Cellulase by Submerged Culture Fermentation

4.1 Production of Crude Cellulase

The term "crude cellulase" refers to the natural cellulase enzyme produced in fermentation, without genetic modification of the microbe and without downstream modifications of the enzymes. The technology for the production of crude cellulase forms the basis for cellulase production involving genetic or downstream modifications. Almost all commercial cellulase produced by submerged fermentation is made by the fungi *Trichoderma, Humicola, Aspergillus,* and *Penicillium*, and this discussion focuses on processes using these microbes.

4.2 Fermentor Operation

Cellulase manufacturers adopt specific procedures for storing and propagating cultures to obtain reproducible fermentations. The cellulase-producing fungi are typically stored frozen at –80 °C or freeze-dried. To prepare the inoculum (seed) mixture, an aliquot is taken and grown in consecutive liquid cultures of increasing volume. The volume of the last step, the seed fermentor, is typically 1–10 % of the main fermentor volume.

Cellulase production is carried out in aerobic, aseptic culture, and many aspects are consistent with that of other such systems used for production of other enzymes or antibiotics. The volume of fermentors used for commercial cellulase production ranges from 95,000 liters to 285,000 liters. The fermentations are highly aerobic, and oxygen is supplied through a sparger at a rate of 0.3 to 1.2 tank volumes per minute. The fermentation vessel is designed and operated to optimize gas transfer and mixing, such as with agitators. Heat generated by the microbial metabolism and agitation is removed through a cooling jacket or coil. Baffles are placed near the wall to increase mixing efficiency and prevent vortex formation. To prevent microbial contamination, the fermentor and support equipment are sterilized before inoculation. Steam sterilization is typically carried out at 121 °C for 20 min. The incoming air is filtered.

The growth medium includes defined salts, complex nutrients, surfactants, and inducer. The salts are the typical fermentation salts, including potassium phosphate, ammonium nitrate, ammonium sulfate, calcium chloride, and magnesium sulfate [33]. The complex nutrients are most often 5 to 25 g L^{-1} of corn steep liquor but can also include yeast extract. The surfactants are added to control or suppress foam formation. The surfactants used include commercial antifoams as well as soybean oil or palm oil. The inducers are proprietary to each manufacturer but will contain an inexpensive mixture of soluble and/or insoluble sugars. Some inducers used include milk whey, which contains lactose; Solka floc cellulose; or, sugar or paper mill waste streams.

The temperature and pH of the fermentor is monitored and controlled throughout the fermentation. For *Trichoderma*, the conditions are 28–30 °C, pH 4–5. For the other fungi, the temperatures and pH are higher, typically 37 °C, pH 6.2–7.

4.3 Progression of Cellulase Production

Most of the literature on the quantitative aspects of cellulase production focuses on *Trichoderma,* as will this discussion.

Figure 3 shows the progression of cellulase production (IU L^{-1} h^{-1}) over the years 1972 to 1989. A 200-fold increase in the productivity of cellulase enzyme

Table 3. Improvement in cellulase enzyme production

Year	Technology	Cellulase Prod'n (IU $L^{-1}h^{-1}$)	Reference
1972	Native strain in batch culture	3	[2]
1974	Selected strain QM9414	10	[34]
1978	Strain MCG77 with continuous culture	32	[35]
1981	Selected strain RutC30	78	[36]
1982	Strain RutC30 in fed batch culture	140	[37]
1984	Strain P37 in fed batch culture	395	[38]
1989	Selected strain in continuous culture	730	[39]

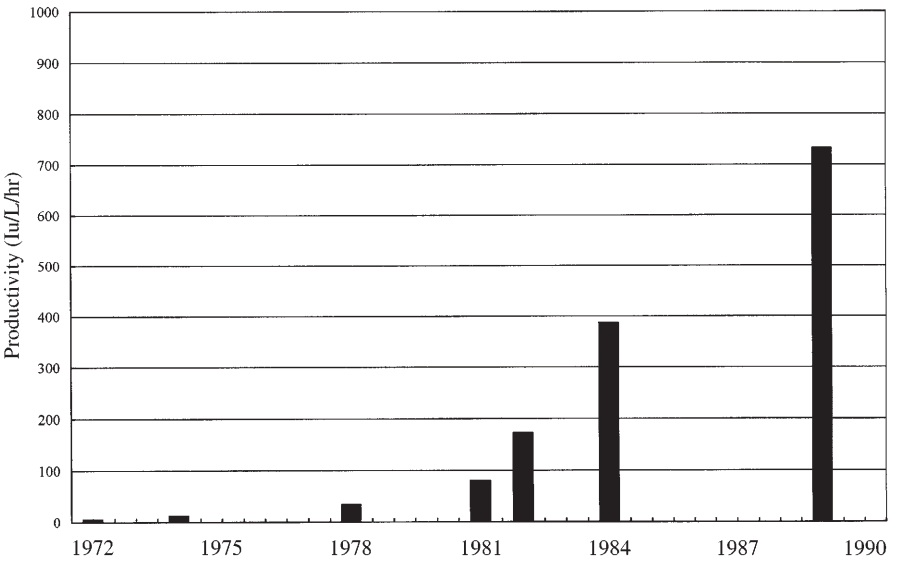

Fig. 3. Cellulase production by *Trichoderma*

production by *Trichoderma* was achieved in this period. The steps in this improvement are listed in Table 3.

This improvement in cellulase production averaged a doubling every 2 years between 1972 and 1984 and resulted from a combination of mutation/selection of strains and the optimization of fed-batch and continuous culture.

The highest rates of productivity reported are those of Nicholson et al. [39]. The results of cellulase production in continuous culture at a dilution rate of 0.018 h^{-1} are summarized in Table 4. Nicholson et al. noted that the maximum cell mass maintained in the 20 liter vessel was 40 g L^{-1} due to foaming, but that this limitation could be overcome in larger equipment.

4.4 Fermentation Kinetics

This commentary is based on cellulase production by *Trichoderma* and is consistent with the general fermentation technology of Wang et al. [40].

Table 4. Cellulase production in continuous culture [39]

Parameter	85 g L^{-1} feed	126 g L^{-1} feed	180 g L^{-1} feed
Cell mass (g L^{-1})	22	33	41
Cellulase (g L^{-1})	22	30	40
Productivity (IU L^{-1} h^{-1})	400	570	730

The general governing equations are as follows:

1. *Growth*: Cell Mass Accumulation equals Growth minus Cell Removal

$$\frac{dX}{dt} = uX - \frac{F_o X}{V} \tag{5}$$

where X is the cell mass, g L^{-1}; t is time, h; u is the specific growth rate, h^{-1}; F_o is the volume removed, L h^{-1}; V is the fermenter volume, L

2. *Enzyme production* (assumes no destruction of enzyme in the fermentor): Enzyme accumulation equals Enzyme production minus Enzyme removal

$$\frac{dS}{dt} = q_p X - \frac{F_o P}{V} \tag{6}$$

where P is the enzyme concentration, g L^{-1}; q_p is the specific enzyme productivity, g (g cells)$^{-1}$ h^{-1}

3. *Substrate utilization* (neglect maintenance energy and substrate removal): Substrate accumulation equals Substrate Feed minus Growth minus Enzyme Production

$$\frac{dS}{dt} = \frac{F_i S_o}{V} - \frac{uX}{Y_x} - \frac{q_p X}{Y_p} \tag{7}$$

where S is the substrate concentration, g L^{-1}; F_i is the feed rate, L h^{-1}; Y_x is the yield of cell mass from substrate; Y_p is the yield of protein from substrate

In addition to the governing Eqs. (5–7), specific information has been reported [37], as follows:

For *Trichoderma* growing and producing cellulase in a medium in which the carbon source is the limiting nutrient, 50% of the carbon is converted to CO_2 by aerobic respiration, and the other 50% of the carbon is taken up as cell mass or protein. Therefore,

$$Y_x = 0.5 \tag{8}$$

$$Y_p = 0.5 \tag{9}$$

The relation between growth rate and substrate concentration can be represented by the Monod model:

$$u = \frac{u_{max} S}{K_s + S} \tag{10}$$

where

u_{max} maximum growth rate, h^{-1}

K_s Substrate constant, g L^{-1}

The relationship between growth rate u and specific rate of enzyme production q_p is shown in Fig. 4 [37]. At the maximum growth rate, there is little protein produced. At decreasing rates of growth, q_p increases, reaching a maximum q_{pmax} at a growth rate that is 60% of u_{max}. Below this rate of growth, q_p remains at q_{pmax}.

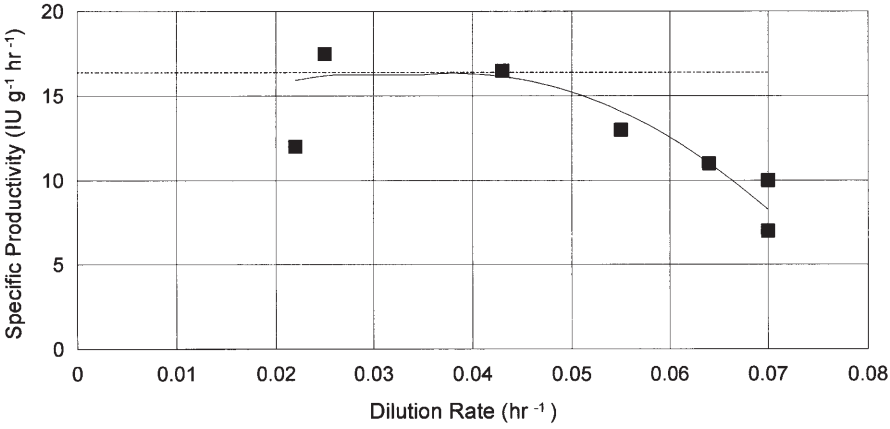

Fig. 4. Enzyme productivity q_p varies with dilution rate

Eqs. (5–10) and Fig. 4 are applied to the design of batch, continuous, and fed batch systems, as follows.

4.5 Batch Culture

In batch culture, Eq. (10) determines the rate of growth. For soluble substrates, during most of the culture, $S \gg K_s$, and therefore $u = u_{max}$. From Fig. 4, there is little protein produced at this rapid growth condition. Batch culture of readily-metabolized substrates is therefore not used to make cellulase with *Trichoderma*.

An interesting alternative, however, is batch culture using cellulose as the feedstock. Because cellulose will only be consumed gradually, the effective value of S is much lower than the actual cellulose concentration, which decreases the rate of cell growth u and, according to Fig. 4, increases q_p. In this system, the substrate uptake rate will depend on the amount of cellulase present. Because of its complexity, the kinetics of fermentation of cellulose will not be developed further here. However, batch culture has been used for cellulase production and is an option to consider in a fermentation design, as the feedstock is inexpensive. Handling and sterilization of the insoluble solid is a concern.

4.6 Continuous Culture

In continuous culture,

$$\frac{F_o}{V} = D \tag{11}$$

where
D = dilution rate, L h^{-1}

In addition,

$$\frac{F_i}{V} = D \tag{12}$$

Combining Eqs. (11) and (5) produces the well known relation between growth rate and dilution rate at steady-state ($dX/dt = 0$):

$$u = D \tag{13}$$

Therefore, continuous culture offers the opportunity to control the rate of cell growth. From Fig. 4, this allows one to select the q_p for high enzyme production.

The steady-state relation between cell mass and dilution rate is obtained by combining Eqs. (5–9):

$$X = \frac{0.5\, DS_o}{D + q_p} \tag{14}$$

The steady-state relation between protein concentration, cell mass, and dilution rate is given by combining Eqs. (6) and (11):

$$P = \frac{q_{px}}{D} \tag{15}$$

Combining Eqs. (14) and (15) relates protein concentration and dilution rate:

$$P = 0.5\, \frac{S_o q_p}{D + q_p} \tag{16}$$

From Eq. (16), the protein production is at a maximum at low dilution rates. This is consistent with [37] (see Fig. 5).

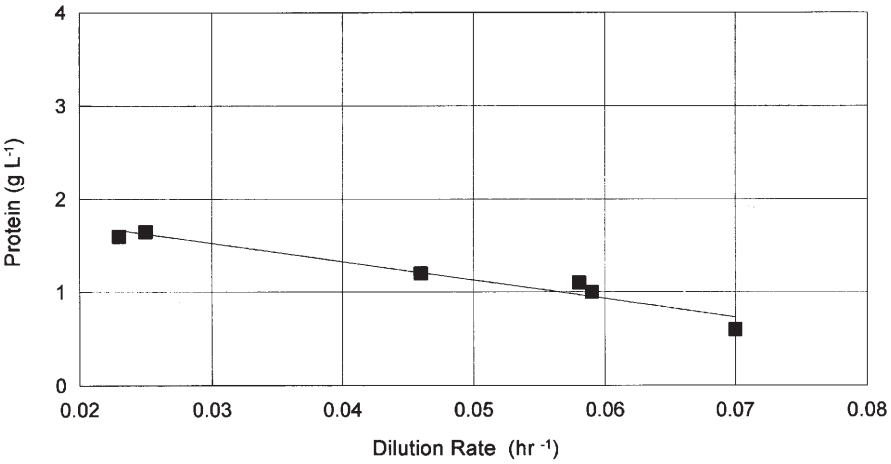

Fig. 5. Protein level varies with dilution rate

In designing a fermentation system, we want to maximize the fermenter productivity, that is, $P \times D$ (g protein L^{-1} h^{-1}). The productivity is given by Eq. (17):

$$P \times D = \frac{0.5\, S_o D q_p}{D + q_p} \tag{17}$$

From Eq. (17), we see that the productivity will be low at low dilution rates, i.e. $D = 0$. The productivity will also be low as D approaches u_{max}, because at this point $q_p = 0$. Therefore, the maximum productivity will be achieved at some intermediate dilution rate. Allen and Andreotti found the optimum at $D = 0.6$ u_{max}, which was the maximum D at which $q_p = q_{pmax}$ (Fig. 6).

4.7 Fed Batch Culture

In fed batch culture, growth rate is controlled by the rate of feeding of the limiting nutrient. The feed rate can therefore be used to set q_p and the desired productivity of protein production.

At a steady state concentration of substrate, Eqs. (7–9) result in Eq. (18):

Equation (18) and Fig. 4 can be taken together to determine three regimes of behavior of fed-batch systems.

$$\frac{F_i S_o}{V} = 2\,(u + q_p)\, S \tag{18}$$

4.7.1 *Overfeeding*

In this case, $2u_{max}X_o/V$. Due to an excess of feed, the growth rate is at u_{max}, and q_p is zero. Using the value from [37] of $u_{max} = 0.075$ h^{-1}, at $X = 4$ g L^{-1} overfeed-

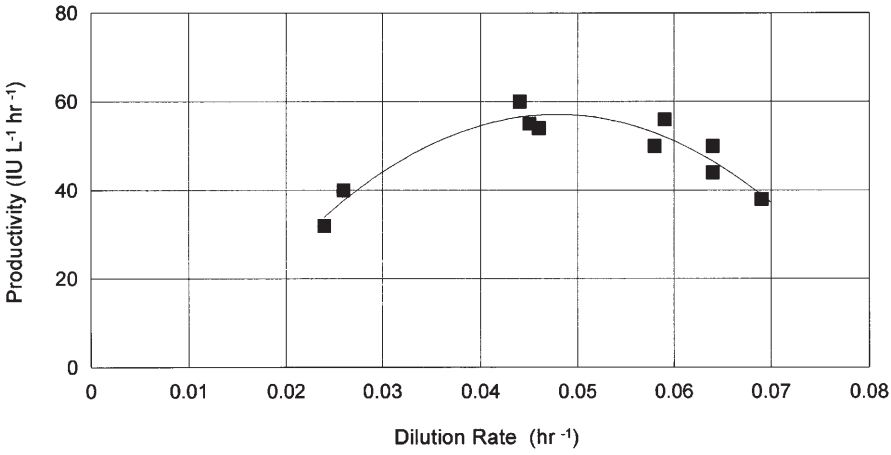

Fig. 6. Productivity varies with dilution rate

ing occurs with $F_iS_o/V > 0.6$ g L^{-1} h^{-1}. This is not a desired operation for protein production.

4.7.2 *High Feeding*

In this case, 2 q_{pmax} $X<F_iS_o/V < 2$ $u_{max}X$. This is a high feed rate such that $q_p < q_{pmax}$ and $u < u_{max}$. The optimum feed rate is determined by integration of Eqs. (6) and (7) over time for fed batch systems, which will not be carried out here. Using the values from Fig. 4, this regime occurs when 0.06 g L^{-1} $h^{-1} < F_iS_o/V <$ 0.6 g L^{-1} h^{-1}.

4.7.3 *Low Feeding*

In this case, $F_iS_o/V < 2q_{pmax}X$. This is such a low feed rate that insufficient nutrients are available to produce protein at a productivity corresponding to q_{pmax}, even in the absence of growth. This regime, which corresponds to $F_iS_o/V <$ 0.06 g L^{-1} h^{-1}, is not preferred for protein production.

The value of q_{pmax} is influenced by the carbon source and the strain.

In terms of the carbon source, glucose is a catabolite repressor, but many oligomers and polymers of glucose induce enzyme production. Lactose, cellobiose, and sophorose are all dimeric sugars that induce cellulase production. Cellulose is also an inducer. A list of the performance of several carbon sources is found in Table 5 [37].

The cellulase productivity varies greatly among strains. Allen and Andreotti [37] reported selection of mutants with 5-fold higher q_{pmax} than the parent strains.

In addition to the carbon source, oxygen can be a limiting nutrient. As described by Wang et al. [40], the ability to transfer oxygen to the cells decreases as the viscosity of the fermentation broth increases. The oxygen requirement (*OTR*) is given by Eq. (19), which takes into account that approximately 50 % of the mass of *Trichoderma* cells and cellulase protein is carbon, and in a fully aerobic state with excess oxygen, all of the oxygen taken up by the cells goes to form CO_2.

Table 5. Performance of carbon sources[a]

Carbon Source	q_{pmax} (mg g^{-1} h^{-1})
Glucose	2.9
Whey (sweet)	8.9
Starch hydrolyzate	9.3
Lactose	16.8
Cellulose BW-200	17.8
Whey (deproteinated)	22.7
Hydrolysis syrup	24.7

[a] *Trichoderma reesei* strain MCG80 in continuous culture with dilution rate of 0.044 h^{-1}.

$$\mathrm{OTR} = 41.67\ (q_p + u)\ X \tag{19}$$

where
OTR Oxygen requirement, mmol O_2 L^{-1} h^{-1}.

The oxygen delivery rate into the system is given by Eq. (20):

$$Na = K_L A\ (C^* - C_L) \tag{20}$$

where
Na oxygen delivery rate, mmol O_2 L^{-1} h^{-1}
$K_L A$ oxygen mass transfer coefficient, mmol O_2 L^{-1} h^{-1} (atm O_2)$^{-1}$
C^* O_2 concentration in inlet air, atm O_2
C_L concentration of O_2 in the broth, atm O_2

In this case, C^* is limited by the pressure that can be maintained in the fermentation vessel. The use of oxygen in place of air will increase Na, but at high cost.

The minimum C_L that will sustain *Trichoderma* is about 20% of the solubility of oxygen in water at 28 °C under 1 atm pressure, that is, about 1–2 ppm.

The relationship between $K_L A$ and fermentor operating variables was given by Cooper et al. [41]:

$$K_L A = C\,(P_g)^A\ (V_S)^B \tag{21}$$

where A,B,C are constants; P_g is gassed power input per unit volume, HP (378 L^{-1}); V_s is superficial air velocity, cm min^{-1}

For a 1200 l continuous culture of *Trichoderma reesei*, Nicholson et al. [39] reported that $K_L A$ varied with the 0.3 power of the power input and the 0.5 power of the superficial air velocity. These values are within 10% of those reported by Cooper et al. [41].

The highest $K_L A$ reported by Nicholson et al. [39] was 320 mmol $O_2 L^{-1}$ h^{-1} (atm O_2)$^{-1}$. At an oxygen pressure of 0.23 atm, a growth rate of 0.04 h^{-1} and with 40 g L^{-1} cell mass, the maximum protein productivity supported by this rate of oxygen transfer is 0.152 g (g cells)$^{-1}$ h^{-1}. This is a significantly higher protein productivity than has been reported, and indicates that, under ideal conditions, a fermentation vessel can deliver sufficient oxygen to the cells.

4.8 Downstream Processing

At the end of the fermentation, protein is separated from cell mass by filtration, typically with a rotary vacuum filter. The crude enzyme concentration is often lower than suitable for commercial applications, so the concentration of enzyme is increased by ultrafiltration. Most cellulase enzymes have a molecular weight of 25,000 to 75,000 and are retained by ultrafiltration membranes of 5000 molecular weight cutoff. The membranes permit the passage of low molecular weight salts, sugars and other impurities, and are sometimes operated in a diafiltration mode to increase the purity of the enzymes. The crude broth at this point is dark brown.

Enzyme formulation is carried out at the fermentation plant, at a separate formulation facility, at the end-users site, or a combination of these. The enzyme is formulated as a liquid or a solid.

For solid formulations, the ultrafiltered broth of 10% to 40% solids content is spray-dried and compacted, or granulated directly [42, 43]. A stabilizer is added during spray drying or granulation. Typical stabilizers are sugars, dextrin, confectionary glaze, and starch; polyvinyl alcohol; clays such as bentonite and kaolin; salts such as sodium chloride crystals or rock salt, sodium carbonate, trisodium citrate. Other additives include citrate or phosphate buffers, to hold the pH of the enzyme when added to water; surfactants such as Triton X-100 to help the performance in textile and detergent applications; binders and coatings, such as polyvinyl alcohol, fatty acid esters, gum arabic and other natural gums, and polyethylene glycol to achieve the desired texture, size, and dust prevention of granulate; colorants, including titanium dioxide to give the products a desirable color; and fillers such as sodium sulfate to achieve the desired enzyme concentration. There is also a small amount of moisture in the solid.

For liquid formulations, stabilizers such as sorbitol, glycerol, or propylene glycol are added to levels of 10% to 50% (w/w) to maintain the activity of the enzyme for up to several months at ambient conditions. Preservatives such as sodium benzoate, potassium sorbate, and sodium chloride are used to control microbial contamination. Buffers and surfactants such as Triton X-100 can also be added, although at lower levels than found in granulated cellulase enzymes due to the limits of tolerance of the proteins for these additives in liquid solutions.

4.9 Cellulase Enzyme Components

The distribution of individual cellulase enzymes in two prominent crude cellulases, those produced by *Trichoderma longibrachiatum* Rut C30 and *Humicola insolens* DSM 1800, are listed in Table 6. Note that the component designations

Table 6. Distribution of cellulase components from representative strains of *Trichoderma* and *Humicola*[a]

Component	*Trichoderma longibrachiatum* RutC30 (%)	*Humicola insolens* DSM 1800 (%)
CBHI	50	20
CBHII	20	< 5
EGI	10	50
EGII	5	5
EGIll	< 5	5
EG5	< 5	10
Beta-glucosidase	1 – 2	1 – 2
Other (non-cellulase)	10	10
Total	100	100

[a] CBH denotes cellobiohydrolase, EG denotes endoglucanase.

do not denote identical enzymes, i.e. *Trichoderma* EGI is not the same enzyme as *Humicola* EGI. Nonetheless, the generalization can be made that the *Trichoderma* cellulase is more heavily weighted toward CBH enzymes.

It is often desirable to enhance or deplete the cellulase enzyme mixture of one or more individual cellulase components. This is done if one or two components are particularly desired, or if one or more are deleterious to the application. In the extreme, only a single cellulase component is produced. In one example of this, pure *Trichoderma* endoglucanase III is produced by extracting it with polyethylene glycol [16].

4.10
Genetic Engineering and Cellulase Production

Through the techniques of recombinant DNA technology, the expression of one or more cellulase components can be enhanced or deleted [44]. Alternatively, a cellulase component can be expressed in a microbe that does not otherwise make cellulase, to make a pure component. An example of this is *Humicola* EG5 expressed in *Aspergillus oryzae* [17]. The resulting pure endoglucanase is free of other cellulase components and more easily controlled in detergent applications.

The cellulase proteins themselves can be modified by genetic engineering. These techniques can be used to remove sections of the cellulase enzymes, such as the binding domains of *Trichoderma* EGI and EGII [21]. Alternatively, specific amino acids can be modified to change the properties of the enzyme, as has been carried out with Humicola EG5. The modified enzymes have increased storage stability in the presence of protease, adsorption to cellulose, stability in the presence of peroxide, and tolerance of anionic surfactants, all of which are advantages in detergent applications [20].

5
Manufacturers of Cellulase by Submerged Fermentation

The following table lists companies that are the largest producers of cellulase by submerged fermentation. There are several smaller producers as well. In addition, many other companies formulate, resell, and/or distribute cellulase.

Company	Location
Genencor International	Rochester, NY
Iogen Corporation	Ottawa, Canada
Novo Nordisk	Bagsvaerd, Denmark
Rohm Finland	Rajamaki, Finland

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Production of Cellulase by Solid-State Fermentation

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The advances in the production of cellulase by solid-state fermentation are reviewed. In the process description of solid-state fermentation for cellulase production, the microorganisms, raw materials for solid-state fermentation, pretreatment of raw materials, sterilization and inoculation, and solid-state fermentation process are included. The effects and control of operating conditions, such as temperature, water content and water activity, pH, aeration, and substrate, are presented. A total of eight types of bioreactors with their advantages and disadvantages for solid-state fermentation are discussed. From the engineering aspect, mathematical models for the solid-state fermentation process are also discussed and several suggestions are proposed which might further research and development of cellulase production by the solid-state fermentation process.

Keywords. Cellulase production, Solid-state fermentation, Operating conditions, Bioreactor, Mathematical model

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1 Introduction

Cellulose is the major constituent of all plant materials and, therefore, is one of the most abundant renewable resources in the world. Most cellulosic materials contain three main components: cellulose, hemicellulose and lignin. Cellulose consists of 3500–10,000 glucose units linked together by 1,4-glucosidic bonds, which makes it a potential raw material in the fermentation industry for the production of energy, food and so on. The challenge is how to hydrolyze the fibrous cellulose into fermentable glucose. In the former Soviet Union, large-scale hydrolysis processes by dilute acid have been developed and the hydrolysate fermented into single-cell protein for animal feed. The process is carried out at around 200 °C and 1.5 Mpa and is energy intensive. Moreover, under high temperature, glucose is further degraded into by-products, such as methyl furfural, which is an inhibitor for microorganism growth. Concentrated acid may also be used to hydrolyze cellulosic materials at mild temperature, but it is difficult to recover the acid for recycling.

In nature, cellulosic materials are broken down by the action of microorganisms. Cellulose is used as a carbon and energy source by numerous bacteria, actinomycetes and fungi. Because of the special type of linkage and biological composition, cellulosic material is the structural material of plant for its strength and rigidity, and these properties make it very resistant to attack by microorganisms. Thus the breakdown rate of cellulosic materials in nature is slow.

It is well known that enzyme cellulases are the reason for the breakdown of cellulose by microorganisms. The search for highly efficient cellulase-producing microbes and methods that can produce cellulase at a low cost are two key

factors for hydrolyzing cellulosic materials into fermentable glucose for commercial application [1].

Intensive research over the last 50 years has led to the identification and isolation of various microbes which are capable of producing cellulases, including myxobacterials, actinomycetales, eubacterials and fungi. Cellulolytic enzymes produced by microorganisms are indeed a mixture of different enzymes with specific functions. Three major types of cellulolytic enzymes are: endoglucanase (1,4-D-glucan-4-glucanohydrolase, EC 3.2.1.4), exoglucanase (1,4-D-glucan-cellobiohydrolase, EC 3.2.1.91) and glucosidase (D-glucosidoglucohydrolase, cellobiase, EC 3.2.1.21) [2].

Most bacteria are incapable of degrading crystalline cellulose since their cellulase systems are incomplete. However, the cellulolytic enzymes produced by some fungi generally involve all three types of enzymes, so they are very useful in the saccharification of renewable cellulosic resources.

Growth of filamentous fungi is an aerobic process. The production of cellulase by fungi can be carried out either by a submerged culture process or solid-state fermentation. In the submerged culture process, a stirred-tank fermentor with temperature, pH, dissolved oxygen (DO) and liquid level control is generally employed and operated in batch mode. The advantages of the submerged culture process include: reproducible enzyme activities in each batch, ease of contamination control, labor saving, etc. The production cost is relatively high because of the high energy requirement, expensive medium composition and low enzyme concentration.

Solid-state fermentation [3–5] is an established process in Asian countries for the production of wine, food additives, etc. It is also applied in the production of organic acid, animal feed and enzymes. If solid-state fermentation is used to produce cellulase, the following advantages and disadvantages should be noted [6–9]:

1. The process is generally labor intensive and hard to control. The reproducibility is relatively poor with batch-to-batch difference. In addition, more care is needed in order to prevent contamination.
2. The raw materials required to produce cellulase are cheap and abundant. Natural cellulosic materials such as plant stems and corn cobs can be used as the carbon source. The composition of the medium is simple and of low cost. Because of the high capability of cellulosic materials to buffer the pH value, it is not necessary to add additional expensive buffer solution.
3. There is no stirrer in most types of solid-state fermentor and the requirements for water and aeration are less than that in submerged fermentation. Therefore, the energy consumption is low and there is no wastewater produced in the process.
4. In solid-state fermentation, the productivity per unit reactor volume is high and the solid cellulase koji can be directly applied to hydrolyze cellulosic materials.
5. The equipment in the solid-state fermentation process is relatively simple and the capital investment is low. However, new types of fermentor for large-scale cellulase production need to be developed.

The most attractive features of solid-state fermentation include low capital investment and low operational costs. These features are favorable in the hydrolysis of cellulosic materials because the cost of cellulase is a key factor for commercial production. Currently, cellulase production by submerged culture has made great progress and been commercialized, while solid-state fermentation is only operated on a small scale in a few countries, such as China, and further efforts in research and development are still needed in order to improve the process and the equipment.

2
Process Description of Solid-State Fermentation for Cellulase Production

2.1
Microorganisms

Various bacteria, actinomyces and fungi can secrete cellulases. Strains that produce cellulases are mainly from *Trichoderma, Aspergillus, Penicillium* and *Fusarium*, in which the most popular strain is *Trichoderma reesei*. Since the 1960s, a series of screening and breeding works have been carried out from a wild strain of *T. reesei* QM6a [2], and various mutants with improved productivity have been obtained as shown in Fig. 1. In addition to the applied strains, the most commonly studied are QM 9414, Rut C30 and MCG 77. These strains can accumulate high activities of endo- and exoglucanase, but are poor in glucosidase [10, 11]. The cellulases from *Aspergillus* strains such as *A. niger* and *A. phoenicis* are poor in the hydrolysis of cellulosic materials but are high in glucosidase activity [11–13]. Strains from both *Trichoderma* and *Aspergillus* can be applied in solid-state fermentation for cellulase production.

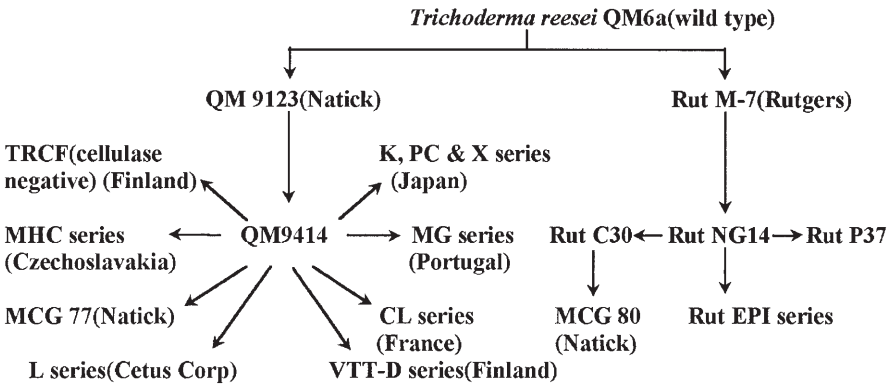

Fig. 1. Worldwide mutant screening program for *T. reesei*

2.2
Raw Materials for Solid-State Fermentation

It is a commonly held view that cellulase is an inducible enzyme, and that cellulose is the best inducer. In solid-state fermentation for cellulase production, cellulosic materials act as either the carbon source or the inducer. Some of the raw materials for solid-state fermentation and their composition are listed in Table 1. From Table 1 it is very clear that the raw materials for cellulase production using solid-state fermentation are abundant and cheap. Proper pretreatment of raw materials is generally required. The nitrogen source for solid-state fermentation includes inorganic compounds, such as urea and $(NH_4)_2SO_4$, or natural products, such as bran.

2.3
Pretreatment of Raw Materials

The crystallinic structure and lignin existence in cellulosic materials prevent attack by microorganisms. The growth of fungi is too slow in untreated raw materials for commercial production of cellulase. In order to design an economically viable technical process, these difficulties must be overcome by a suitable mechanical or chemical pretreatment.

An efficient pretreatment must set free the highly crystalline structure of cellulose and extend the amorphous areas. The removal of lignin is also essential. A decrease of one-third in the lignin content of hardwood or two-thirds in that of softwood increases the digestibility of these materials to 60%. The requirement for pretreatment in solid-state fermentation is not as great as that for submerged fermentation or enzymatic hydrolysis. Some agricultural residues, such as bran and corn cob, and industrial waste, such as recycle paper and cellulosic residue from the xylose manufacturing process, can even be used without pretreatment.

Table 1. Composition of cellulosic raw materials for solid-state fermentation of cellulase

	Corn stove	Wheat straw	Rice straw	Rice hulls	Bagasse fiber	Cotton gin trash	News-print	*Populus tritis*	Douglas fir
Carbohydrate (% sugar equivalent)									
Glucose	39.0	36.6	41.0	36.1	38.1	20.0	64.4	40.0	50.0
Mannose	0.3	0.8	1.8	3.0	na	2.1	16.6	8.0	12.0
Galactose	0.8	2.4	0.4	0.1	1.1	0.1	na	na	1.3
Xylose	14.8	19.2	14.8	14.0	23.3	4.6	4.6	13.0	3.4
Arabinose	3.2	2.4	4.5	2.6	2.5	2.3	0.5	2.0	1.1
Non-carbohydrate (%)									
Lignin	15.1	14.5	9.9	19.4	18.4	17.6	21.0	20.0	28.3
Ash	4.3	9.6	12.4	20.1	2.8	14.8	0.4	1.0	0.2
Protein	4.0	3.0	na	na	3.0	3.0	na	na	na

na: not available.

2.3.1
Pulverization [14]

Pulverization can reduce the size as well as the crystallinity of cellulosic materials and increase the surface area and bulk density. It is also possible to separate part of the lignin from carbohydrates which makes it easier for microorganisms to digest cellulose. Various equipment, such as a compression mill, a bead mill, an extruder, a roll mill and disc refiners, etc., can be used for pulverization. Unfortunately these methods tend to be very expensive and too energy intensive. For solid-state fermentation, if the particles are too fine, the oxygen mass transfer will become a big problem; therefore, lightly crushed or just ground raw material will suffice.

2.3.2
Alkali Treatment

Alkali treatment of cellulosic materials can remove lignin as well as part of the hemicellulose and expose cellulose for fungi to digest. In addition, the alkali treatment method can swell and disrupt the structure of cellulose. Commonly used alkalis includes NaOH, $Ca(OH)_2$ or CaO and aqueous as well as gaseous NH_3. For solid-state fermentation, after alkali treatment, the cellulosic materials do not need to be washed, because all the solubilized lignin and hemicellulose can be retained in the raw material [15]. After the addition of other nutrients, the pH has to be adjusted to a value of approx. 6.5 with H_2SO_4.

2.3.3
Dilute Acid Prehydrolysis

After dilute acid treatment most of the hemicellulose is removed from the cellulosic materials. The other functions of dilute acid prehydrolysis include the increase in surface area and pore volume. However, the crystallinity of the cellulose changes little [16]. Dilute acid treatment is carried out at 110–220 °C and 0.3–1.1 % acid concentration. If the acid treatment is performed at elevated temperature, pentosans may be converted into furfural, which is an inhibitor for most microorganisms.

2.3.4
Steam Explosion

Cellulosic material is treated with steam at 200–240 °C for between about 30 s and 20 min then withdrawn from the reactor quickly to create an explosion. The result is that hemicellulose is decomposed into low-molecular-weight, water-soluble products and lignin is also degraded; both effects are favorable for digestion [17–21]. Steam explosion has been commercially applied in Canada [22]. The expensive equipment, high energy requirement and the production of furfural in the process are the drawbacks of this technique.

A similar technique is to use ammonia or carbon dioxide instead of steam as the carrier gas for explosion. The process is performed at moderate pressure (15 atm) and mild temperature (50–80 °C) [17, 23] and the ammonia or carbon dioxide can be recycled.

2.4 Sterilization and Inoculation

2.4.1 *Sterilization*

The raw materials for solid-state fermentation should be sterilized at 121 °C. The sterilization is carried out in situ or in vitro dependent on the fermentor type and scale. In order to enhance the heat transfer in the solid material, the medium pile should not be too tight and direct steam is generally employed. The heating time is in the range 30–60 min. For in vitro sterilization, a fixed bed, fluidized bed, rotating drum or belt sterilizer, etc. can be used.

2.4.2 *Inoculation*

The seeds for solid-state fermentation are prepared either by submerged culture or solid-state culture. Bran is an excellent medium for solid seed preparation. After the spores have matured, the solid seeds are harvested and mixed with the pre-sterilized medium for solid-state fermentation. The ratio between solid seeds and medium is around 1:20–50.

The liquid seeds are prepared in a stirred-tank fermentor with an air supply. For *T. reesei*, a suggested medium composition [24] is as follows (g/l): glucose 10, pepton 1.0, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, urea 0.3, $CaCl_2 \cdot 2H_2O$ 0.4, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.05, $MnSO_4 \cdot H_2O$ 0.016, $ZnSO_4 \cdot 7H_2O$ 0.014 and $CoCl_2 \cdot 6H_2O$ 0.037. The cultivation conditions are 30 °C and pH 4.8. After 48 h, liquid seeds are harvested and mixed with the medium in a ratio of 10% (v/v).

2.5 Solid-State Fermentation Process

The operating conditions for solid-state fermentation for cellulase production are dependent on the strain to be used, the reactor type and the medium composition, but the basic operating procedure remains the same as shown in Fig. 2. The final product can be obtained as crude solid cellulase, liquid cellulase or powder cellulase according to the application. Figure 3 shows a process flowsheet for cellulase production [25]. In the process, wheat bran is used as substrate. Seeds are prepared in a stirred-tank fermentor and then sprayed into the medium by a spray distributor. The fermentation is performed in a shallow-tray fermentor. The temperature and humidity in the fermentor are automatically regulated. After fermentation, cellulase is recovered by water extraction and purified by salt precipitation and ion exchange. The final product is concentrat-

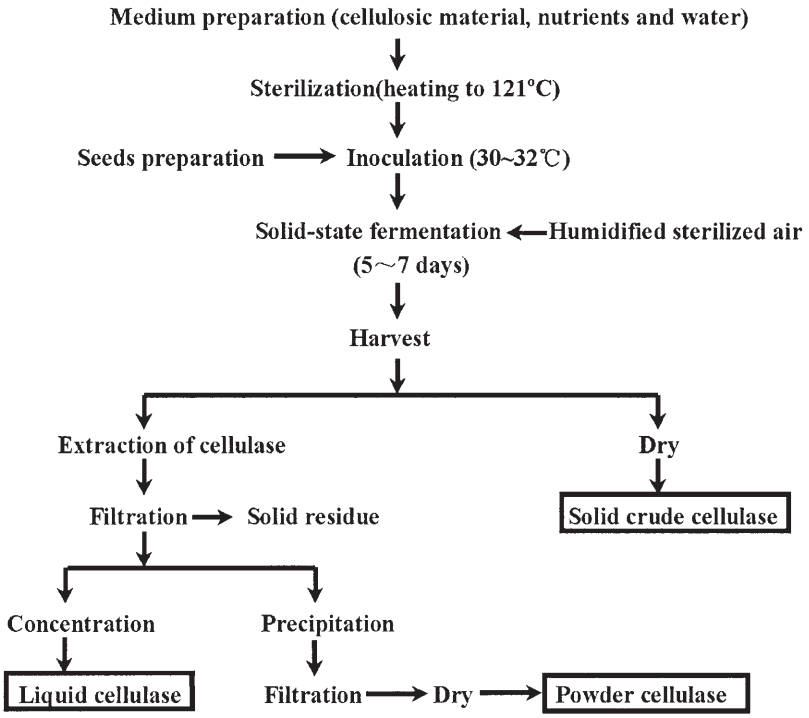

Fig. 2. General flowsheet for cellulase production via solid-state fermentation

ed by ultrafiltration and is then spray-dried. If the requirement for the quality of the cellulase is not too high, the final product may be obtained as solid koji or precipitated after salt precipitation.

3 Operating Conditions for Solid-State Fermentation

The operating conditions are very important for efficient cellulase production via solid-state fermentation. The effects of operating conditions, such as temperature, water content and water activity, pH, aeration and substrate composition, on the cellulase production by solid-state fermentation will be discussed in this section.

3.1 Temperature

The most commonly used strain in cellulase production is the mutant from *T. reesei*, whose optimal temperature for growth is in the range 30–32°C, and the optimal temperature for cellulase accumulation is 26–28°C [2, 26]. During solid-state fermentation, a heat of metabolism will be released because of the

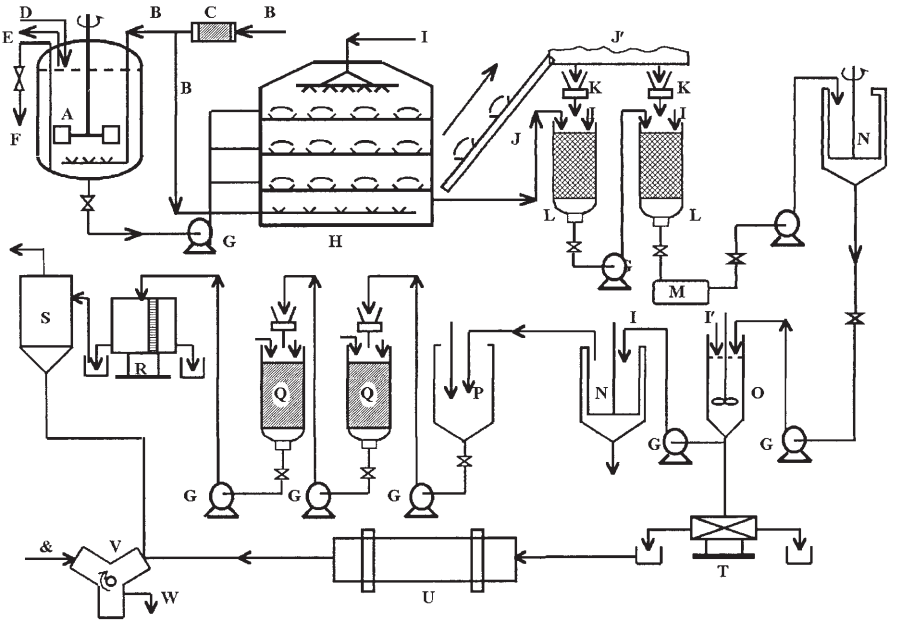

Fig. 3. Process flowsheet for cellulase production in wheat bran culture [25]. *A* Submerged seed culture of *T. viride*; *B* oil-free compressed air; *C* air filter; *D* inoculum; *E* exhaust air; *F* sample collection; *G* centrifugal pump; *H* automatic wheat bran culture of *T. viride*; *I* water spray; *I'* ammonium sulfate, alcohol and water; *J* conveyor belt; *J'* screw conveyor; *K* hopper; *L* extraction column; *M* storage tank; *N* centrifuge; *O* precipitation; *P* mixing tank; *Q* ion-exchange column; *R* membrane concentrator; *S* spray dryer; *T* filter press; *U* rotary dryer; *V* mixer; *W* cellulase preparation and salt stabilizer

vigorous growth of the fungi. The heat transfer in the solid layer is relatively poor and overheating is possible in the center of the solid substrate. This is unfavorable for spore germination, mycelial growth and enzyme accumulation. The death of mycelia and subsequent failure of fermentation are possible because of severe overheating. Temperature control in the environment of the solid-state fermentor is relatively convenient to achieve, but temperature regulation within the solid substrate layer is not so easy. The following methods may be employed to control the temperature in the solid phase [27–29]:

1. A proper thickness of solid phase is favorable to facilitate the heat transfer;
2. Regulation of the aeration rate to fulfill the requirement for both oxygen mass transfer and heat transfer. Forced convection should be performed if necessary; and
3. In the fermentor design, batch or continuous stirring of the solid phase should be considered if possible.

3.2
Water Content and Water Activity

Controlling the water content in the solid substrate is the key factor for successful cellulase production. Water is an essential component in the solid medium for spore germination, mycelial growth and cellulase production. If the water content is too high, the void space, as well as the gas-phase volume within the solid substrate, are reduced, which increases the mass transfer resistance of oxygen and carbon dioxide, as well as the possibility of contamination. Low water content is unfavorable to spore germination and substrate swelling. Substrate swelling is essential for fungi to attack and to digest the solid substrate. The optimal water content, that is changeable dependent on the strain and the fermentor, is in the range 50–80% [30–32].

Kim et al. studied the solid-state fermentation processes of *T. reesei* and *Sporotrichum cellulophilum*. The optimal water content for growth phase of *T. reesei* and *S. cellulophilum* is 57 and 70%, respectively, but is the same as the cellulase production phase (50%) [31].

Indeed, water activity is a more important factor than water content for fungi growth and metabolism [29, 33–37]. Water activity gives the amount of unbounded water available in the immediate surroundings of the microorganism. Water activity is closely related, but not equal to, the water content. The definition of the water activity is:

$$a_w = P_s/P_o \tag{1}$$

where P_s is the equilibrium vapor pressure of water within the solid substrate and P_o is the vapor pressure of pure water. The optimal water activity for eukaryotes and prokaryotes is 0.7 and 0.9, respectively [38]. There are exceptions; for example, for *T. viride* TS mutant, the optimal water activity for growth and spore formation is 0.99 and 0.98, respectively [39]. Small perturbations of water activity around the optimal value will greatly affect the growth and metabolism of microorganisms.

In the solid-state fermentation process, the water activity is variable because of evaporation and metabolism. In order to keep the water activity constant, the humidity of the supplied air should be controlled or a humidifier should be installed in the fermentor. The relationship between the water activity within the solid substrate and the equilibrium relative humidity (ERH) of the surrounding air can be expressed as:

$$a_w = m_w/(m_w+m_i) = \mathrm{ERH}/100 \tag{2}$$

where m_w and m_i are the number of moles of water and of all solutes, respectively [27].

3.3
pH

The pH value is an important operational parameter in the submerged fermentation of cellulase. During solid-state fermentation it is difficult to measure and

control the pH value. If $(NH_4)_2SO_4$ is used as the nitrogen source, the pH value of the medium will decrease with the consumption of NH_4^+ by the microorganisms. The decrease in the pH value can be matched if 40–50% of urea is used in the nitrogen source. A complex nitrogen source is also favorable for fungi growth and cellulase accumulation [40]. At the same time solid cellulosic substrate is a good buffer for pH change and therefore it is not necessary to adjust the pH value during solid-state fermentation. The initial pH value of the solid medium should be in the range 4.5–6.5 [15, 41].

3.4 Aeration

The solid-state fermentation for cellulase production is an aerobic process; therefore, aeration is necessary for cell growth and cellulase formation. In addition, aeration is important in order to maintain humidity, to remove gaseous metabolites, such as CO_2, as well as to remove the heat of metabolism [42, 43]. In a large-scale solid-state fermentor, aeration is so important that it must be controlled carefully. The aeration method and the flow rate as well as the temperature and humidity of the air supply must be regulated to avoid sudden changes in temperature and water activity of the solid medium [29, 31].

Liu et al. found that discontinuous aeration was a good alternative. In their process, if the temperature reached 34°C, aeration was switched on and off if the temperature dropped to 29°C. The humidity of the air was kept at 90% of saturation. After 56–60 h, the cellulase activity reached the maximum value [44].

3.5 Substrate

In the submerged fermentation process for cellulase production, crystal cellulose and paper pulp are commonly used substrates which are expensive. The solid-state fermentation for cellulase production should use a cheap medium. Because cellulase is an inducible enzyme, the inducer must be included in the medium. Cellulose is the best inducer [45, 46] and its hydrolysate (glucose) is the carbon source for microbes to grow; therefore, cellulosic material is the basic component in the medium. Complex nitrogen sources are used to supply nitrogen and to regulate the pH value. Other nutrient salts, such as Mn^{2+} and Zn^{2+}, etc., should also be involved in the medium.

Wheat bran, which contains cellulose, protein and other nutrients, is an excellent medium for solid-state fermentation [25, 47]. Wheat bran is also widely used in other fermentation industries but is still too expensive for economic cellulase production. Agricultural residue is cheaper. For example [30, 48], after pretreatment by 4% NaOH at 121°C for 0.5–1.0 h, wheat straw was used as raw material. After mixing with nutrient salts, adding water to a water content of 80%, then adjusting the pH to 5.8, the medium was used for solid-state fermentation. The final cellulase activity was reported to be as high as 200–430 FPIU per gram of cellulose. Deschamps et al. [49] used a mixture of wheat straw and bran (80:20) as raw materials for solid-state fermentation; the

cellulase activity was 18 FPIU per gram of initial substrate. Schamala and Screekantiah [50] utilized rice straw as the raw material for economic production of cellulase and xylanase. Wang et al. compared different substrates, such as rice straw, wheat straw and corn stove, for solid-state fermentation of cellulase and found that corn stove was the best substrate [51]. In our laboratory, corn cob, without any pretreatment except for crushing into 3-mm particles, was used as substrate. After adding bran and other nutrients, the solid-state fermentation was carried out in a deep-trough fermentor for 1 week; the cellulase activity reached 110–180 FPIU per gram of dried koji.

From the above discussion it is obvious that agricultural residue can be used as substrate and inducer in solid-state fermentation for economic cellulase production. This is a distinguished advantage of solid-state fermentation and makes the process more competitive than the submerged culture technique.

4
Bioreactors for Solid-State Fermentation

As mentioned before, the solid-state fermentation process is generally labor intensive and hard to control. The reproducibility is relatively poor with batch-to-batch difference and, in addition, it is difficult to prevent contamination. In order to improve solid-state fermentation, the key point is to design a solid-state fermentor which performs well. Compared with the fermentors used for submerged culture, the research and development for solid-state fermentor is underdeveloped. A well-designed solid-state fermentor should (1) have perfect control systems for temperature, air flow rate and humidity; (2) have a well-designed system for preventing contamination; (3) be homogeneous in water activity, temperature and composition so that microbes can grow uniformly; (4) be able to remove harmful metabolites, such as CO_2, quickly; and (5) be labor saving and easy to scale-up for handling solid medium. Currently used solid-state fermentors cannot meet all of the above requirements.

4.1
Shallow-Tray Fermentor

There is a long history in China and other countries of using shallow-tray fermentors for solid-state fermentation. The shallow tray can be made of wood, bamboo, metal or plastic. The bottom of the tray is a sieve plate or wire mesh to facilitate air flow and to hold the solid substrate. The height of the tray is approx. 30–50 mm. A suitable space should be left between the trays. An array of trays is installed on a specially designed support, which is located in a sterilizable room to prevent contamination. Sterilized air is blown into the room supplying oxygen for microbes to grow. The humidity and the temperature in the room are controllable.

The advantages of the shallow-tray fermentor are the simplicity of its construction and the low investment involved. In a shallow-tray fermentor, the oxygen transfer in the solid bed occurs primarily by diffusion instead of forced convection [29]. The thickness of the solid substrate layer in the tray is, there-

fore, limited to within the range 30 – 50 mm to maintain the uniformity of solid substrate in temperature, water activity and oxygen transfer. In addition the tray fermentor needs a large operational area. Although the activity of cellulase in the final koji from a shallow-tray fermentor is high, the productivity is low and the labor requirement is generally intensive.

Figure 4 shows the shallow-tray fermentor used by Kim et al. [31]. Air saturated with water vapor was blown into the bottom of a culture vessel, and the exhaust air was withdrawn at the top. Similar apparatus has been employed by various research groups to produce cellulase in a small-scale, pilot-plant and commercial production facility [52 – 57].

4.2 Column Fermentor

A column fermentor is a fixed-bed reactor. Solid medium is put into the column with entries at both ends for aeration [58 – 60]. Figure 5 shows an experimental apparatus developed by Saucedo-Castaneda et al. [28]. Sterile air can be supplied either by a radial or axial gradient method. The water activity is maintained by humidified air. The temperature for the solid fermentation is monitored and controlled by recycling water in the jacket from an isothermal bath. In a fixed-bed column fermentor, the oxygen transfer and CO_2 dissipation are im-

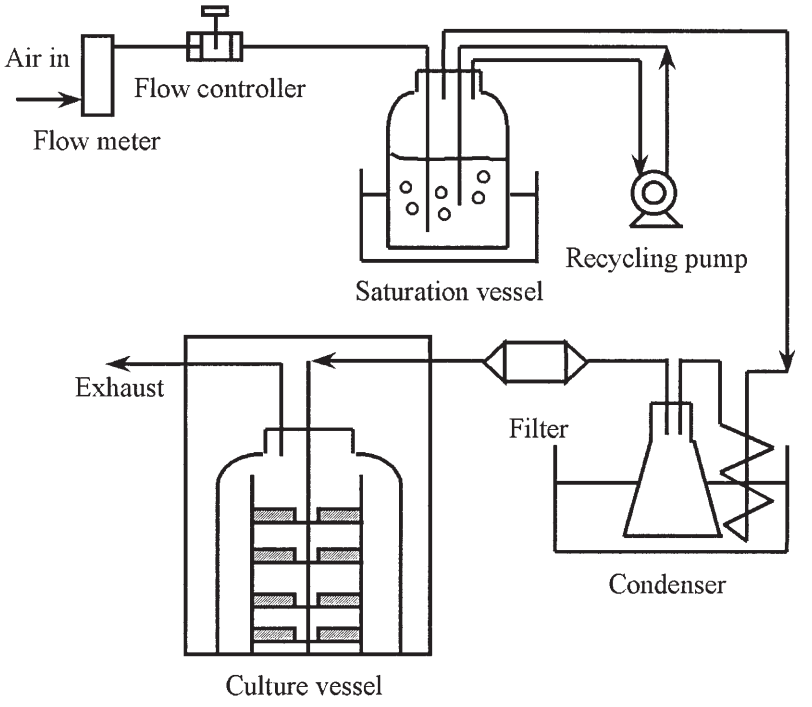

Fig. 4. Schematic diagram of a solid-state cultivation system for cellulase production [31]

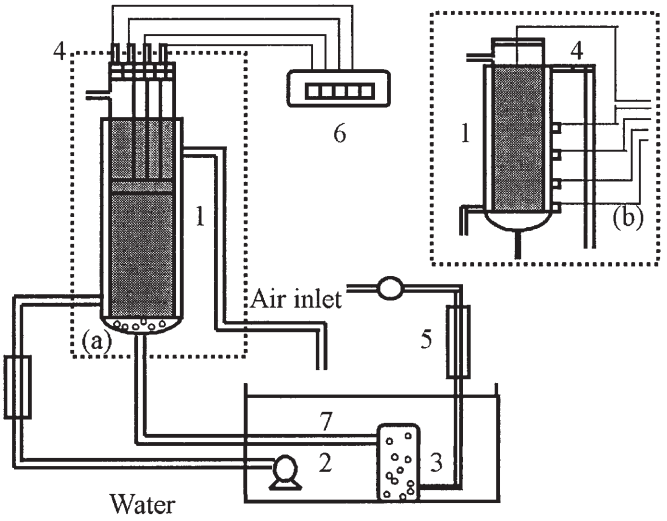

Fig. 5. A column fermentor for the monitoring of the temperature gradient in solid-state fermentation of cassaca by *A.niger* [28]. **a** Radial gradient; **b** axial gradient. Column fermentor (volume: 1 l; radius: 6 cm; length: 35 cm): *1* jacket fermentor; *2* water pump; *3* humidifier; *4* thermocouples; *5* pressure and air flow controls; *6* temperature display device; *7* water bath

proved by forced convection. It is, however, difficult to regulate the water activity and temperature in the fixed bed. In addition, handling the solid materials in a column fermentor is a huge problem and scale up to a column fermentor is troublesome.

4.3 Deep-Trough Fermentor

The deep-trough fermentor is used for deep-bed fermentation with forced aeration. It consists of a rectangular koji compartment and an air-water regulation compartment as shown in Fig. 6 [52].The typical size of the koji compartment is about $4 \times 2 \times 1.5$ m. The thickness of the solid substrate layer is 20–40 cm. The bottom of the koji compartment is a sieve plate to allow air blowing. The temperature and the humidity of the air are regulated in an air-water regulation compartment. The forced air convection enhances the oxygen mass transfer and removes metabolic heat and CO_2 from the bed. In addition to cellulase production, the deep-trough fermentor is used for enzyme production, food fermentation and wine manufacture in eastern Asian countries [57, 61]. The deep-trough fermentor is able to produce cellulase on a large scale with high productivity and uniform quality. However, as fermentation progresses, the substrate shrinks because of mycelial growth, which causes a reduction in bed porosity and affects oxygen transfer and temperature gradient in the solid substrate.

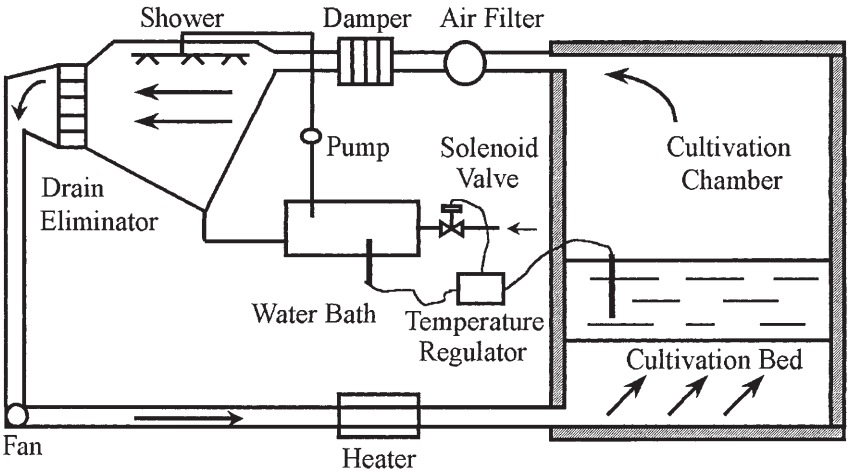

Fig. 6. Deep-trough fermentor with forced aeration [52]

4.4 Rotating-Drum Fermentor

The rotating-drum fermentor consists of a drum-shaped container mounted on a roller which acts as a support and rotating device. The solid substrate in the drum will move up and fall down again with the rotation of the drum. Forced aeration is employed through an inlet pipe with holes in it. The inlet pipe may lay parallel to the bottom or branch at several points of the drum [32, 52]. Figure 7 shows the rotating-drum fermentor used by Tao et al. [62].

There are various types and applications of the rotating-drum fermentor [3, 63–65]. Hrubant et al. developed a three-chambered drum fermentor with buffles for production of Ochratoxin by *Aspergillus ochraceus*. Inoculated solid substrate is added in the first chamber. With a slow rotation of the drum, the fermentation medium is gradually moved forwards in the axial direction. Finally, koji is harvested in the third chamber. The fermentation process is operated continuously [66]. Lindenfelser and Ciegler [67] designed a fermentor with four simulated cross sections of a 33-cm diameter buffled drum. The four drum sections were spaced on a common shaft made of stainless steel pipe. The shaft had four holes in it to allow a passage of air to be forced through the hollow shaft. Han and Anderson [68] used a 30-gallon capacity cement mixer, rotating at 1 rpm, for straw fermentation using *Trichoderma viride, Candida utilis* and *Aureobasidium pullulans*. It was found that microorganisms grow rapidly during the period from 18–40 h after incubation.

With the rotating-drum fermentor, the oxygen mass transfer is enhanced and overheating is prevented. The heterogeneity of the system is also reduced to a large extent as compared with static trays or a packed-bed fermentor [29]. It is possible to operate a rotating-drum fermentor continuously which enhances

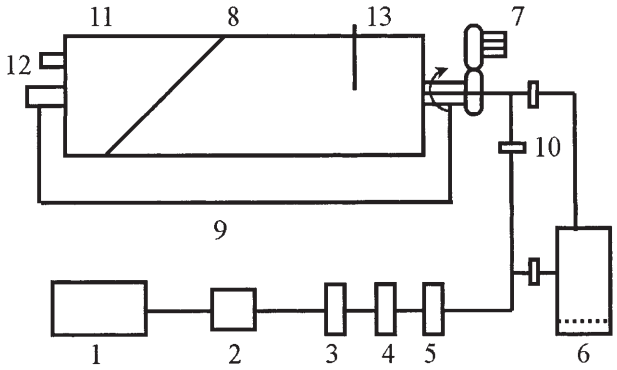

Fig. 7. Rotating-drum fermentor [62]. *1* Air compressor; *2* pressure regulation valve; *3* oil separator; *4* air filter; *5* air heater; *6* atomizer; *7* rotating device; *8* rotary-drum fermentor; *9* fermentor stand; *10* gas valve; *11* fermentor cover; *12* air outlet; *13* thermister

the productivity and reduces the labor requirement. However, there are several disadvantages associated with a rotating drum fermentor:

1. The tumbling of the solid substrate during fermentation in a rotating drum may break mycelium, thereby hindering growth especially under high rotating speed [52, 69].
2. The aggregation of substrate particles into balls may occur which will cause growth inhibition.
3. Low volumetric productivity (the useful space in the drum is only about 30% of the total) [5].

4.5 Stirred-Tank Reactor

The stirred-tank reactor consists of a box or tank with a stirrer in it. The solid medium in the tank is stirred in batch or continuous mode to facilitate oxygen mass transfer. Figure 8 shows the rectangular pilot reactor (2 × 0.8 × 2.3 m) designed by Durand and Chereau [70] to culture *T. viride* with sugar beet pulp as raw material. The thickness of the substrate layer in the reactor is 1 m. Three vertical screws are bound to a conveyor with dual direction movement. The linear movement speed of the conveyor and the rotating rate of the screws are 6.5 cm/min and 22 rpm, respectively. With this specially designed agitation device, new surfaces are introduced to aeration without much break up to the mycelium and the aggregation formation of substrate is avoided. An air-conditioning system and continuous monitoring system for process parameters were integrated to ensure the fermentation process.

Deschamps et al. [49, 71] applied a stirred-tank reactor of 65-l capacity to produce cellulase by *T. harzianum*. Aeration was provided by passing humidified air through the perforated bottom of the tank. When the temperature reached above the set point, occasional agitation and water spraying were put

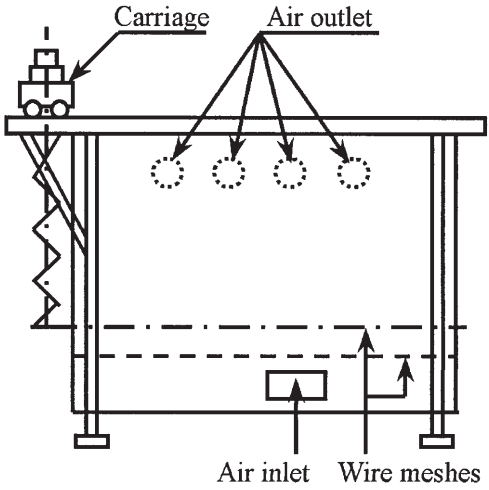

Fig. 8. Schematic of a reactor and agitation device [70]

into action. Baldensperger et al. [72] applied a stirred-tank reactor with a capacity of 15 kg (dry weight) for solid-state fermentation of banana wastes to produce cattle feed. The reactor was a modification of a bread-making blender, and its base was perforated for steaming and aeration.

Compared with the rotating-drum reactor, the stirred-tank reactor is easy to operate, and the aggregation of substrate to form particles can be avoided. Because the substrate is quite wet, the energy consumption for agitation is generally high added to which the stirred tank reactor is hard to isolate completely from the environment and, therefore, contamination may occur.

4.6
Rotating-Disk Reactor

The Fujiwara Brewing Machinery Company of Japan has developed a piece of equipment for continuous koji production which is shaped like a rotating or circular plate. When the plate rotates in the horizontal plane, the solid substrate tumbles about, thus contacting the air which enters through the holes in the plate [52, 73–74].

4.7
Rocking-Drum Reactor

In order to avoid over disturbing the solid medium and to prevent inhibitory effect on the growth of microbes, Ryoo et al. [75] developed a new reactor, i.e. the rocking drum reactor. As shown in Fig. 9, the reactor is in slow rocking motion. During the fermentation process, the solid substrate is gently rocked so that the air supply and humidity can be uniformly distributed. A computer-con-

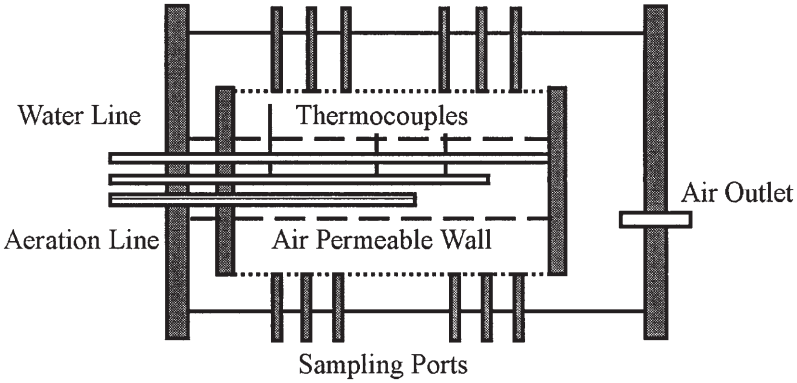

Fig. 9. Rocking-drum reactor for solid-state fermentation [75]

trolled system is applied to regulate temperature and humidity. The air enters in the center of the drum, then passes through the solid substrate and air-permeable wall. The relative humidity of the air is regulated according to the temperature measurement. The water loss due to evaporation is supplemented by spraying cold water into the substrate to keep the water activity constant. Because of the excellent control system, high biomass productivity can be achieved. Disadvantages of the rocking-drum reactor are the high costs to manufacture the equipment and to maintain the complex control system. The economic feasibility of the rocking-drum reactor should be evaluated further [29].

4.8 Fluidized-Bed Fermentor

Gas-solid fluidized-bed bioreactors are actively used to perform solid-state fermentation [74, 76]. The particular solid substrate is placed on a porous plate or metal net and sterilized air is blown in under the plate. When the air flow rate is high enough, solid substrate particles will be suspended in the gas phase. Advantages of the fluidized-bed bioreactor include:

1. The solid substrate particles mix with air perfectly so that the oxygen mass transfer can be improved greatly.
2. Mycelia damage caused by mechanic stirring is avoided.
3. Uniform distribution of temperature and humidity in the bioreactor is achieved.
4. Compared with traditional solid-state fermentation, the period of fermentation is shortened and the productivity is increased.
5. The solid substrate is easy to handle in a fluidized-bed bioreactor. The fermentation is carried out in an isolated vessel to avoid contamination.

Akao and Okamoto [77] applied a fluidized-bed bioreactor to produce koji enzymes by *Aspergillus oryzae* with higher biomass productivity and enzyme

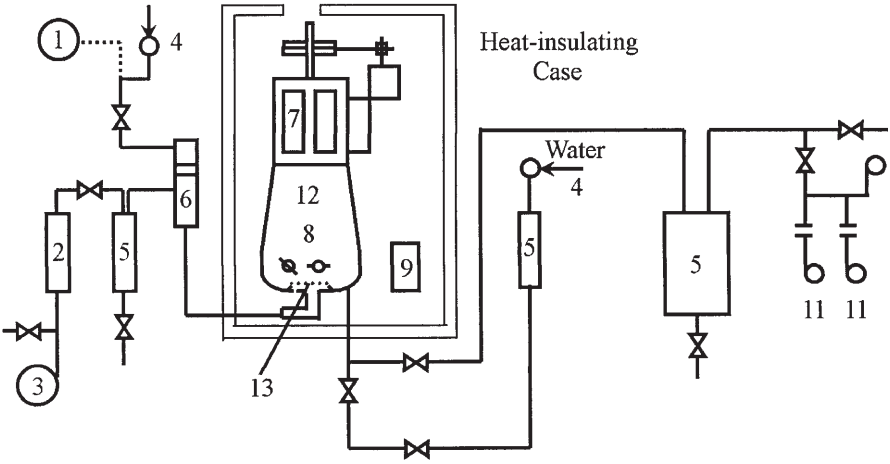

Fig. 10. Schematic of batch air-solid fluidized-bed fermentor [78]. *1* boiler; *2* flow meter; *3* blower; *4* water pump; *5* air filter sterilizer; *6* heater; *7* separator; *8* agitators; *9* fan heater; *10* nozzle for spraying humidified air into the fermentor; *11* air compressor; *12* fermentor; *13* distributor

activity. Tanaka et al. [78] used a gas-solid fluidized-bed bioreactor with a stirrer in the solid substrate entrance to culture *Eupericillium javanicum* for amylase and protease production. A vapor stream rather than water spraying was used to humidify the air. The apparatus is shown in Fig. 10. The amylase activity obtained in this fermentor is four times higher than that in a traditional fermentor. Crooke et al. [74] revised the gas-solid fluidized-bed fermentor with a different design. Application of the fluidized-bed bioreactor in cellulase production is still lacking and needs further evaluation.

5
Mathematical Model for Solid-State Fermentation

A mathematical model is essential for process optimization and scale up. For solid-state fermentation, setting up a mathematical model is a much more difficult task than for the submerged culture process because of the difficulties in parameter measurement and the complexity in mass transfer reaction interaction.

5.1
Parameter Measurement

Parameters to describe a solid-state fermentation process for cellulase production include: physical properties such as temperature, pH, humidity and water activity, particle size, pile and real densities, pore size and porosity, etc.; chemical components such as medium composition, gas-phase composition, amount and properties of microorganisms, activities of cellulases, etc.; and kinetic para-

meters such as diffusivity, mass and heat transfer constants and various rate constants for bioreactions.

The measurement of physical properties is relative easy and can be carried out on-line and/or off-line. Temperature, pH and water activity should be measured and controlled around a set point. Because the solid substrate is consumed by microorganisms, the particle size, pile and real densities, pore size and surface area must be measured off-line and will change with time.

The chemical compositions are all time variable. The gas-phase composition can be measured on-line by an infrared analyzer or mass spectrometer. The composition of the solid substrate must be analyzed off-line by a chemical method. It is very difficult to measure the biomass amount in solid-state fermentation processes, especially for filamenteous fungi. Several indirect methods, such as estimation of biomass based on glucosamine determination, on ergosterol and total sugar, on DNA assay, and on on-line monitoring of carbon dioxide evolution, as well as direct methods such as use of the light scattering technique to estimate biomass in the presence of solid particles and on-line measurement based on infrared analysis, etc., have been developed to estimate the biomass amount during solid-state fermentation processes [29, 79 – 80]. The cellulase activity during solid-state fermentation may be assayed by the following procedure: first extract the cellulase from the solid koji with water under stirring and then assay the activity of the cellulase in aqueous solution by standard methods [81]. There are different methods to represent the activity of cellulase, such as filter paper unit (FPIU), CMC-ase and-glucosidase activity etc., depending on the application of the cellulase.

The oxygen mass transfer is very important in solid-state fermentation and the following factors should be considered: concentration gradient within the solid layer as well as in the solid particles, gas-liquid equilibrium, dissolved oxygen (DO)-solid phase (cell mass) equilibrium and the mass transfer rate as well as oxygen uptake rate. The dissipation rate of carbon dioxide should also be measured. Heat is generated by metabolism and may be measured by a calorimetric method or estimated by various empirical equations. The heat transfer in a bed consisting of porous particles is hard to measure and to estimate. Most of the metabolic heat is removed by the gas flow and, therefore, the heat transfer between the solid phase and the gas phase must be estimated. The substrate consumption rate and the cellulase formation rate can be calculated from the measurement of the solid-phase composition and enzyme activity at certain time intervals. The growth rate of cell mass is estimated from the measurement of biomass. For a more detailed model, such as a structured model or a morphological model, information about cell composition and morphology must be offered. Some of the kinetic parameters have to be correlated from the experimental data.

5.2 Mathematical Model

A complete mathematical model to describe a batch solid-state fermentation for cellulase production in a fixed bed should include the following:

1. Mass transfer equations for oxygen and carbon dioxide;
2. Heat generation and heat transfer equations;
3. Kinetic equations for biomass growth and death;
4. Equations for substrate consumption rate;
5. Equation for cellulase formation rate.

A solid-state fermentation is a multiphase system and the homogeneity in the solid phase is generally imperfect making it more difficult to model the process.

Mass and heat transfer during fermentation are very important but hard to describe. For mycelial microbes such as *T. reesei*, the increase in biomass is contributed by tip extension and branching and mycelial pellets are formed. The size, porosity and density of the particulate solid substrate will change after consumption. The mechanism of the interaction between microbes and solid substrate is not yet fully understood. Therefore, various simplification assumptions must be made to represent the solid-state fermentation processes.

In the solid-state fermentation, water exists only in the porous substrate and mold. For an aerobic process, oxygen should be dissolved into water then taken up by the mold hyphae. A pore diffusion model is generally applied to represent the oxygen concentration gradient in mold pellets and a mass transfer equation derived from double-film theory is employed to describe the oxygen transfer from the gas to the liquid phase. If oxygen is not a limiting substrate for cell growth, oxygen mass transfer can be neglected in the mathematical model.

The temperature gradient in solid particles is negligible because of the small size and slow bioreaction rate. Sangsurasak and Mitchell [82] developed a two-dimensional dynamic heat transfer model for a cylindrical-bed solid-state fermentation process with pseudo-homogeneous assumption. A logistic growth equation was adopted to describe cell growth in which the first-order death kinetics were included and the model parameters were related to temperature. The temperature distribution was represented by a partial differential equation considering axial air flow and radial heat conduct in the bed. Substrate consumption and product formation were not included in the model.

Because biomass is generally measured by indirect methods for solid-state fermentation, growth kinetics based on carbon dioxide evolution, glucosamine accumulation, nutrient uptake and enzyme production, etc., have been developed. Ramana Murthy et al. [29] have summarized the equations in the literature in a review paper. Smits et al. [83] studied the influence of temperature on kinetics in solid-state fermentation with *T. reesei*. Fermentations of wheat bran were carried out in Petri dishes containing only 5 – 7 g of sterilized solid substrate at different temperatures; therefore, a homogeneous solid phase was assumed. The radial growth rate, maximum specific growth rate, oxygen consumption rate and CO_2 production rate were correlated with temperature. The dry substrate weight loss was related to CO_2 production rate. The specific CMC-ase activity was calculated by determining the amount of CMC-ase activity per amount of biomass glucosamine present.

In a model developed by Kim et al. [31], a shallow-tray fermentor for producing cellulase with *T. reesei* QM9414 and *Sporotrichum cellulophilum* was em-

ployed with temperature and humidity controllers. They found that the cell growth rate was correlated to both oxygen uptake rate and the intracellular glucosamine content. Also a definite correlation between the specific oxygen consumption rate and the specific growth rate at varying water content was found. The increment of cellulase produced was correlated with the water content, cell mass and total amount of oxygen consumed.

Compared with submerged fermentation, the mathematical model for solid-state fermentation is still underdeveloped.

6 Concluding Remarks

Solid-state fermentation is an important method for producing cellulase with a wide range of substrate, low energy consumption and without waste water. The produced cellulase koji may be directly applied to hydrolyze cellulosic materials into fermentable sugar. The enzymatic hydrolysis can also be coupled with the fermentation process (simultaneous saccharification and fermentation, SSF) which is favorable to eliminate product inhibition during enzymatic hydrolysis. Because the fermentation product, such as ethanol and single cell protein, from cellulosic materials generally has a low selling price, solid-state fermentation should be a good choice to produce cellulase at low cost. A strain of microorganism with a high ability to produce cellulase is highly desirable. Genetic engineering is the new direction to obtain new strains. Except for the screening of high productivity strains, from an engineering point of view, the following aspects should be emphasized in further research and development for solid-state fermentation:

1. Pretreatment technology for cellulosic materials for commercial production of cellulase by solid-state fermentation for various raw materials with low cost;
2. Highly efficient and economic sterilization technology for medium preparation in large quantity;
3. Research and development of a bioreactor for large-scale solid-state fermentation that is less labor intensive and has low costs.
4. Development of new methods for the measurement of various operational parameters to control and optimize the process of solid-state fermentation; and
5. Further study of the mass and heat transfer, the kinetics of substrate consumption, cell growth and product formation, establishment of a workable mathematical model for process scale up and optimization for solid-state fermentation.

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Dilute-Acid Hydrolysis of Lignocellulosic Biomass

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In recent years, treatment of lignocellulosic biomass with dilute sulfuric acid has been primarily used as a means of hemicellulose hydrolysis and pretreatment for enzymatic hydrolysis of cellulose. A significant advancement has also been made in the area of dilute acid hydrolysis of cellulose. An overview of reactor theory as it applies to the dilute acid hydrolysis and recent developments in the process technology are discussed. The focus of the new development is the emergence of a counter-current shrinking-bed reactor. In a counter-current reactor scheme, the major portion of the sugar is produced near the liquid outlet point. This minimizes the residence time for the sugar to decompose. Bed shrinking occurs due to partial solubilization of the solid biomass. A properly designed shrinking-bed reactor can reduce the liquid throughput thus raising the sugar concentration. A broader range of reaction conditions has also been explored. These activities contribute to a phenomenal improvement in the dilute acid hydrolysis technology placing it to the point where it can compete with the enzymatic process.

Keywords. Dilute acid, Pretreatment, Hydrolysis, Kinetics, Reactor design

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1 Introduction

Saccharification of cellulosic biomass by dilute acid has a much longer history than the enzymatic process. Acid-catalyzed wood saccharification was in operation in Germany as early as the 1940s [1]. A series of pilot-scale investigations on wood sugar production was also carried out in the USA by the Forest Product Laboratory and TVA shortly after World War II [2, 3]. A number of commercial-scale wood sugar plants producing ethanol and other products such as furfural and yeast cells were in operation in various parts of the old Soviet Union. In recent years, however, treatment of lignocellulosic biomass with dilute sulfuric acid has been primarily used as a means of pretreatment for enzymatic hydrolysis of cellulose. The conventional dilute acid based cellulose hydrolysis has become an unpopular concept, if not totally abandoned, ever since it was known to pose an inherent technical problem. This stems from the fact that sugars are decomposed under the severe conditions needed to promote cellulose hydrolysis, i.e. high temperature and low pH. Despite the diminished interest in acid technology, there has been a continual research effort on the subject. As such, a number of different reactor configurations have been proposed and investigated. They include plug-flow reactors [4], percolation reactors [5], progressive batch/percolation reactors [6], counter-current and co-current reactors [7].

There have also been new developments in kinetic investigation. The impetus of the new kinetic work came from exploration of a broader range of reaction conditions in terms of the temperature and acid concentration [8]. The reactor development work has been contiguously pursued both in hemicellulose hydrolysis [9] and cellulose hydrolysis [10]. Novel ideas have evolved and have been incorporated into new reactor design and simulation studies. These fundamental studies have led to a series of laboratory-scale process studies at the National Renewable Energy Laboratory (NREL). The recent development along these lines has progressed to the point where the acid hydrolysis technology has now become a viable alternative in the biomass saccharification process.

In this paper, we provide an overview of the reactor theory as it applies to the dilute acid hydrolysis and an assessment of the recent development in the process technology. While this paper gives an overall review, emphasis is placed on theory and practice of the biomass reactors performing dilute acid saccharification. Issues concerning the future development of this technology are also discussed.

2 Kinetics

A brief overview of the reaction kinetics concerning the acid hydrolysis of cellulose and hemicellulose is presented here for subsequent discussion on reactor design and operation.

2.1 Cellulose Hydrolysis

The reaction pattern of acid hydrolysis of cellulose by dilute acid is represented by the sequential first-order reaction:

$$\text{Cellulose} \xrightarrow{k_1} \text{Glucose} \xrightarrow{k_2} \text{Decomposed products}$$

The rate constants are represented by an Arrhenius equation in which the pre-exponential factors includes the acid concentration term to account for the effect of acid as well as the temperature:

$$k_i = k_{0i}[A]^{ni} \exp(-E_i/RT) \quad (1)$$

where i = 1 (hydrolysis reaction), i = 2 (decomposition reaction), n_i = acid exponent, [A] = concentration of sulfuric acid, E_i = activation energy.

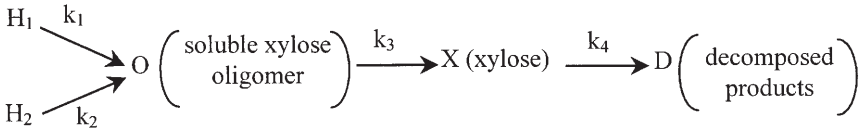
This equation has been applied to most of the kinetic studies and found to give reasonable levels of agreement with experimental data [11, 12]. Sulfuric acid has been the predominant choice for the established processes. A few laboratory studies have been made with other types of acids (phosphoric, nitric). The advantages go in favor of sulfuric acid in cost and corrosion aspects (in comparison to hydrochloric acid). A wide range of experimental conditions has been applied in terms of the temperature and the acid concentration. The reported kinetic parameters of Eq. (1) vary considerably. This is because studies reported in the literature use different substrates and sample preparation methods. The conditions employed to initiate and quench hydrolysis vary widely. Acid loading dependence is accounted for in different ways. Most importantly, the studies overlook the neutralizing ability of biomass on the effective acid concentration. Yet there is one common fact that holds true in most of the kinetic investigations. It is that the activation energy is greater for the hydrolysis reaction than the decomposition reaction. High reaction temperature thus favors the hydrolysis more so than the decomposition. The yield of glucose therefore increases with the reaction temperature meaning that in practice the highest possible temperature should be applied. The upper limit of this temperature is restricted only by practical factors such as the reactor pressure and the ability to control the short reaction time.

In the old processes used until the 1960s, concentrations of 0.5–2.0% sulfuric acid and temperatures of 170–200 °C were the common range of the reaction conditions applied, thus referred to as "dilute acid process". With renewed interest in the process in the early 1980s, temperatures above 200 °C were explored in various laboratory investigations. This perhaps is a reflection of advancement in experimental techniques that can cope with precise control of short reaction time, high pressure, and corrosion conditions. Since the late 1980s kinetic investigations covering temperatures up to 230 °C have appeared. These investigations accompanied extremely low acid levels. The low acid level is compensated by the high temperature condition in keeping the reaction time within a controllable range. In regard to this condition, Baugh and McCarty [13] found an important kinetic behavior. The decomposition of sugar is basically pH dependent (acidity). In this work, however, a surprising observation was made: that the decomposition of glucose reaches a minimum at or near a pH of

2.5. At higher pH, the decomposition increases again indicating that the reaction is not solely dependent on the acidity. Perhaps other factors start to take effect such as OH-induced reaction. An extremely low acid level of 0.05–0.1% was applied to retain the pH near 2.5. Most of natural biomass feedstock exhibits a certain level of buffering capacity [14, 15]. Therefore the actual pH does not exactly correspond to the acid input when subjected to high temperature and acidic conditions. The follow-up study on the kinetics under extremely low acid (henceforth abbreviated to ELA) concentration was made by Mok et al. [8]. The unusual behavior of the decomposition reaction with regard to pH was further verified experimentally. The "projected" glucose yield at 212 °C in a flow-through reactor was highest (surpassing 80%) at a sulfuric acid concentration of 5 mM equivalent to 0.05 wt%. At acid levels higher than this, the yield of glucose decreased. The maximum observed glucose yield in this study using a flow-through type reactor was 60–70% at 215 °C. This yield is substantially higher than those obtainable under the common conditions previously applied (~1.0%, 190 °C). The experiments in this study were made with pure cellulose (Whatman filter paper) and it disputes the normal kinetic pattern of the sequential two step reaction. Nonetheless, it forms a milestone in the kinetic work that a reaction condition previously unexplored gave rise to a substantial improvement in the acid hydrolysis process. The concept of using ELA was further investigated at NREL in a series of kinetic and process development studies using hardwood substrates [10]. The results of these studies reaffirmed the kinetic advantages of the ELA condition than the moderately low acid condition for obtaining higher glucose yields.

2.2 Hemicellulose Hydrolysis

At high temperatures, the kinetic pattern of hemicellulose hydrolysis can be expressed in a manner similar to that of cellulose hydrolysis (i.e. sequential first-order reactions in series). At lower temperatures ($T < 160$ °C), however, hemicellulose hydrolysis is not homogeneous. There is a portion that hydrolyzes rapidly while the remainder hydrolyzes more slowly [16]. Grohmann et al. [17] characterized this inhomogeneity for hardwoods in the form of two parallel pseudo-first-order reactions, one describing a fast-hydrolyzing fraction, and the other describing a slow-hydrolyzing fraction. The fraction of slow-reacting xylan is estimated to be 0.20–0.32 [14, 15, 17]. The reaction pattern of the acid hydrolysis of hemicellulose at low temperatures can be described as shown below. The kinetic pattern of hemicellulose hydrolysis, H_1, refers to the fraction of hemicellulose easy to hydrolyze and H_2 refers to the fraction difficult to hydrolyze.



Kinetic pattern of hemicellulose hydrolysis: H_1 refers to the fraction of hemicellulose easy to hydrolyze, and H_2 refers to the fraction difficult to hydrolyze.

During the initial phase of the reaction process, random attack by acid on the hemicellulose chains produces oligomers with varying degrees of polymerization. The oligomers are hydrolyzed to monomers, which under normal hydrolysis conditions are degraded further to decomposition products. The overall hydrolysis reaction is, in fact, a continual depolymerization process in which the average molecular weight is gradually decreased. The intermediate product, oligomer, is arbitrarily defined as the water-soluble polymer ($1 < DP < 10$) [18]. The oligomers obtained during the reaction process have different degrees of polymerization (DP). The hydrolysis rates of oligomers vary with the DP value. The hydrolysis rate at the non-reducing end group is greater than the other bonds [19]. The concentration of the various oligomers of different DP values cannot be measured accurately and are usually much smaller than monomeric concentrations. For these reasons, most kinetic studies exclude oligomers from their models.

Kim and Lee [14] have reviewed the kinetics of acid-catalyzed hydrolysis of hemicellulose and provide a useful tabulation of previously published kinetic rate constants. There is wide variation in reported rate constant values, reflecting again the differences in the substrate and pretreatment conditions, the model employed and the method by which the effective acid concentration (or weight percent) was determined [16]. Unfortunately, only a few researchers have performed detailed kinetic studies in which both hydrolysis and decomposition rates are determined. Kim and Lee [14] have carried out the only study including both the fast and slow xylan fractions and xylose decomposition.

Biomass species differ significantly in their ability to neutralize acids. Kim and Lee [14] estimated the amount of acid neutralized from results of a previous neutralization study. Maloney et al. [15] considered not only the neutralization capacity of biomass but also the reduced dissociation of sulfuric acid at high temperature. Correcting for both factors yielded a neutralization capacity of 3.5 g sulfuric acid/kg biomass (white birch). The exponents on acid concentration determined using corrected acid concentration values were very close to 1.0, being 0.93 ± 0.05 and 1.02 ± 0.16 for the fast- and slow-reacting xylan fractions, respectively [16]. This indicates that dependence on effective acid concentration is roughly linear. The exponents on acid concentration reported in the literature range from about 0.5 to 1.5, this is probably because most researchers have neglected to correct acid and/or the neutralizing capacity of biomass [5, 20].

The heterogeneity in hemicellulose hydrolysis (i.e. consisting of fast- and slow-hydrolyzing fractions) has been explained in a number of different ways as to why one part of hemicellulose can be easily eliminated from cell walls whereas the other is more firmly bound. First, a part of the hemicellulose is located in the cell wall in such a way that it is easily accessible to the action of the reagent, whereas the other part is located at a greater depth, and is firmly retained between cellulose chains. Second, this difference in the properties of xylan is caused by the difference in the polymeric structure of the xylan as acetyl and uronic acid ratios to xylose change. Third, a portion of the xylan could be intimately associated with the lignin matrix by being embedded within that matrix or linked to the lignin by lignin–carbohydrate bonds [21]. The

biphasic nature of hemicellulose, however, brings about a number of interesting points concerning the reactor design and operation, especially with regard to the temperature policy and flow configuration in the reactor system and these issues are addressed in the next section.

3
Reactors and Processes in Acid Hydrolysis of Lignocellulose

3.1
Batch Reactor

Batch reactors have been frequently employed in kinetic investigations of acid hydrolysis of cellulose. A sample of projected profile of glucose based on the classical kinetic parameter of Saeman [11] is shown in Fig. 1. The calculated profiles reflect the fact that the maximum yield of sugar increases with temperature. This is in agreement with the kinetic finding that the activation energy of the hydrolysis reaction is higher than that of decomposition. The maximum yield in the batch reactor shown in the figure is only about 40% at 210°C, the heart of the problem in dilute acid hydrolysis. Application of higher temperature, although it would increase the yield in theory, is simply not feasible because of operational difficulties originating from rapid reaction rate. The calculation details are more complicated in the case of hemicellulose hydrolysis because of the more complex reaction pattern. We refer to Chen and Lee [22] for

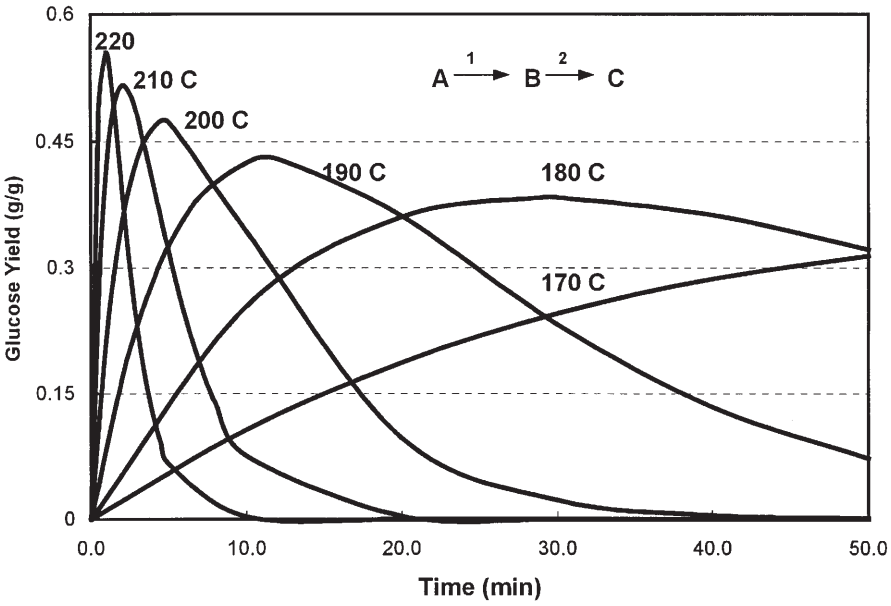


Fig. 1. Glucose yield as a function of time. Yield is calculated using the rate constants of Saeman (1945), 1 wt% sulfuric acid

the analytical solution in hemicellulose hydrolysis in a batch reactor. The sole parameter determining the maximum yield is the ratio of the two kinetic constants, k_2/k_1 (henceforth denoted as α). The value of α for cellulose hydrolysis under a representative reaction condition is in the vicinity of 1.0. The same ratio for hemicellulose hydrolysis under its representative reaction condition is about an order of magnitude lower. The maximum yield of pentoses (primarily xylose) obtainable from batch reaction of hemicellulose hydrolysis thus reaches the 80–85% range. It is the primary reason why dilute acid processing has been a popular choice as a pretreatment method for enzymatic hydrolysis.

3.2 Plug-Flow Reactor

The continuous version of the batch reactor is a plug-flow reactor (PFR). This is a flow-through reactor in which the liquid and the solid travel through the reactor at the same velocity. Although the operation and design of a PFR is quite different from a batch reactor, the modeling processes of the two are identical. The batch reactor equations are therefore directly applicable for a PFR if the time variable (t) is replaced by the residence time ($\tau = L/u$). Two types of laboratory PFR designs have been investigated for the dilute acid hydrolysis of cellulosic biomass. They have been used for kinetic as well as process studies. Thompson and Grethlein [4] introduced a PFR for kinetic study on hardwood (Douglas fir), Kraft paper and Solka-Floc. The system has a tubular reactor through which the biomass slurry is pumped by an external positive displacement pump. An electrical preheater is used for the temperature control. An important feature of this system is that the reaction is initiated by the injection of acid at the entrance of the reactor. With this design, precise control of residence time at low range (less than 1 min) is feasible. More importantly it allowed the study on rapid reactions that occur at high temperature range. The kinetic work of Thompson and Grethlein [4] explored the experimental conditions covering 0.5–2.0% sulfuric acid at 200–240 °C. The maximum yield of glucose obtained from this system was about 50% for Solka-Floc and newsprint and occurred at 230 °C, 1% sulfuric acid, and 0.22 min of residence time. It is a significant improvement to a straight batch system where the maximum yield is only 40% as previously mentioned. The improvement is primarily due to the difference in maximum allowable temperature between a batch reactor (210 °C) and a short residence time PFR (230 °C). The same research team later developed a steam-injected PFR and used it for the kinetic investigation of corn stover cellulose [23]. With this system, they were able to reduce the residence time further down to 6 s elevating the temperature to 240 °C. Glucose yield from corn stover in the range of 55–58% was obtained in this work. The same type of reactor was employed for a study on decomposition of glucose at high temperature with short residence time [24].

Another example of a short residence time PFR is that of New York University [25]. It has a different design in the reactor segment in that a twin-screw extruder is used as a moving-bed mechanism eliminating the external slurry pump. Although the detailed design in acid injection and temperature

control system are different, the reaction conditions and the residence time used for this reactor system are close to those of Thompson and Grethlein [4]. This system was developed as a process reactor rather than a kinetic reactor. As such, a number of process studies have been made on saccharification of various substrates. Green et al. [26, 27] used this reactor for saccharification of municipal solid waste containing 37–50% glucan content. Processing at a pH of 0.5 and a temperature of 230–235°C with 8–15 s of residence time, they reported a maximum glucose yield of 60%. Waste paper (irradiated and hydro pulped) and sawdust have also been investigated as the feedstock for this process [28, 29]. Under similar conditions (232°C, 10–20 s), they obtained 50–60% glucose yield for both substrates. The same paper reports the importance of the sugar concentration of the end product. The reactor discharge of a PFR is a slurry or wet biomass. Additional solid–liquid separation is required to recover the sugar in liquid form. In order to retain high sugar concentration in the products, it is necessary to use a dense, low water feed into the reactor. Rugg et al. [30] contend that their twin-screw extruder reactor can accommodate solid feeds with a wide range of solid/liquid ratio (10% for waste paper pulp, 95% for sawdust).

These high-temperature PFRs offer certain advantages in that they are continuous and have short residence time. The high-temperature condition enhances the yield of glucose from a batch reactor. While the improvement in yield by 10% (from ~50 to ~60%) is significant, it is far too low in comparison to the enzymatic process and from a process viewpoint. Can then an even higher temperature be applied to further improve the yield? The answer is no. There are two main factors that prevent it: (1) Difficulty in controlling the residence time in the range of a few seconds, especially for a large reactor system; and (2) heat transfer limitation (uneven reaction) within the biomass particles that reduces the yield of sugars [31]. Because of these inherent problems, it is doubtful whether a PFR-type reactor will ever find its place as a process reactor for cellulose hydrolysis. However, it may find a worthy application for hemicellulose hydrolysis where the kinetics is much more favorable for saccharification. As evidence, Rugg et al. [30] reported an overall sugar yield of 80% in their work on two-stage continuous hydrolysis of hardwood sawdust using the twin-screw reactor.

3.3 Percolation Reactor

A percolation reactor is a packed-bed flow-through reactor. A conceptual sketch of this reactor is given in Fig. 2, along with two additional reactor types which are discussed later in this section. There are certain advantages in this reactor in comparison to a straight batch reactor or a PFR. First, the sugar product is removed as it is formed. This provides an important benefit in that it reduces sugar decomposition. Second, a packed-bed reactor can be operated with a high solid/liquid ratio. Therefore, a relatively high concentration of the sugar can be obtained. Third, unless the feedstock is in extremely fine particles, the liquid product is separated as it leaves the reactor. A solid/liquid separation is not ne-

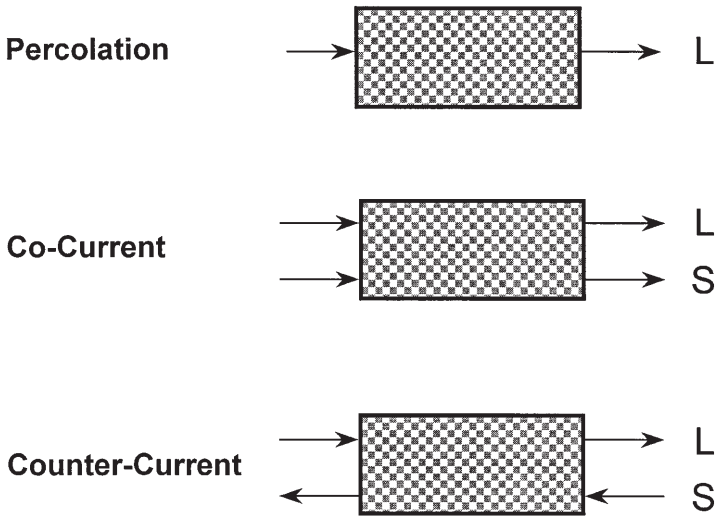


Fig. 2. Reactors used in the acid hydrolysis of lignocellulose

cessary as it would be in a batch reactor or a PFR. It is a reactor relatively easy to operate since it does not involve a moving-bed mechanism inside the reactor.

Although percolation reactors have been in use extensively over several decades, it was not until 1983 that the first theoretical model of this type of reactor was introduced [5]. The model was developed for sequential first-order reactions in order to assess the performance in hydrolysis of hemicellulose. As an unsteady reactor, the model involves a partial differential equation with the following parameters: kinetic parameter: $\alpha = k_2/k_1$; operational parameter: $\beta = k_1 L/u$, $\tau = ut/L$, where L is the bed length and u is the liquid flow velocity.

The process variables of interest are the yield and the concentration of the product (sugar). They are expressed as function of α and τ only, β being a parameter optimized for a given set of α and τ. The parameter τ is an entity proportional to the total liquid throughput. For example, $\tau = 1$ indicates that the liquid has reached the exit point of the reactor. Likewise $\tau = 2$ indicates one reactor volume of liquid has been recovered from the reactor. If the reactor is operated with $\tau = 4$ (three reactor void volume of liquid is to be collected), then how fast the liquid is fed into the reactor is an important issue as it will affect the reactor performance. Too slow feeding will cause severe sugar decomposition, too fast feeding will leave the biomass unreacted. The parameter β comes into effect here as an optimization parameter involving the flow rate. The projected performance of a percolation reactor based on the model of Cahela et al. [5] is shown in Fig. 3 by a plot of the yield vs. product concentration for various sets of τ and α values. In the operation of this reactor, the yield generally increases with τ (amount of liquid throughput), whereas at the same time the product concentration decreases. If this reactor is operated with a high liquid

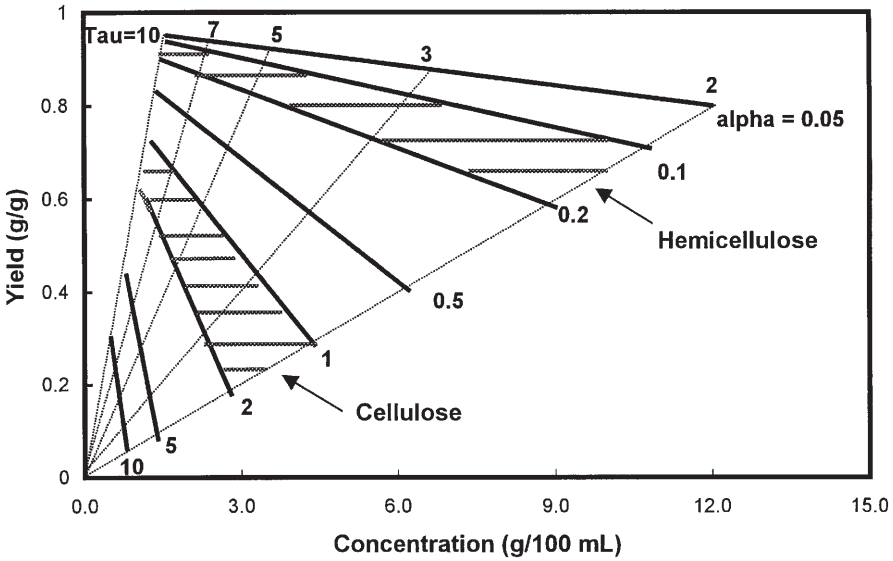


Fig. 3. Yield vs. concentration in a percolation reactor

throughput, a high yield of sugar is obtained but it is done at the expense of the sugar concentration. An inverse relationship thus exists between the yield and the product concentration as shown in Fig. 3. The higher the concentration, the less processing cost and energy would be required in the product separation phase. A trade-off thus exists between the two factors. The point is that the yield value alone without the corresponding concentration is meaningless as a performance indicator. The plot of Fig. 3 (yield vs. concentration) is a simple and the most appropriate way of presenting the reactor performance. The figure has two shaded regions representing the hemicellulose and the cellulose in biomass as they are subjected to dilute acid hydrolysis in a percolation reactor. Taking a point where the concentration is 3%, the figure indicates that the yield for hemicellulose is upward of 90% whereas that for cellulose it is only 50–60%.

The percolation reactor is the one that was used in most of the old wood sugar processes such as the Scholler process [1], the Madison wood sugar process [32, 33], and some of the Soviet processes. Although each process has some unique features in the process details, they all employed a reactor that falls under the category of percolation reactor. For example, in the Scholler process, the reactor was run in a repeated batch manner. For one batch of solid feed, the liquid was replaced several times. The overall reaction mode is similar to the percolation process except that the liquid is fed intermittently, rather than continuously. In all of these processes, the reported yield and sugar concentrations are in the range of 45–55% and 2–4 wt% respectively, in general agreement with the predictions of Cahela et al. [5] (Fig. 3). In one laboratory investigation [34], yields of 80–87% were reported in hydrolysis of wood using a cyclone reactor. The operation mode of this reactor is similar to a percolation reactor in that the

residence times for solid and liquid are varied, the liquid traveling much faster than the solid. However, the high yields were obtainable only with dilute sugar concentration because of the high liquid throughput in the cyclone reactor.

3.4 Hemicellulose Hydrolysis/Pretreatment

With the emergence of cellulase enzymes and their application as saccharification catalysts in the early 1980s, dilute acid hydrolysis has been primarily used as a method of pretreatment for enzymatic hydrolysis. Lignocellulosic materials are only partially digestible in their native form. Increasing their digestibility is of great concern in the enzymatic process. The two factors widely believed to control enzymatic digestibility are the accessible surface area and the crystallinity of the substrate. Increased accessibility of cellulose enables more enzymes to bind to cellulose fiber surfaces, whereas decreased crystallinity increases the reactivity of cellulose, i. e. the rate at which bound enzyme hydrolyzes glucosidic linkages. One common trait of the dilute-acid treatment of lignocellulosic biomass is the increase of porosity and overall surface area. This is achieved by complete or partial removal of hemicellulose and/or lignin. Disruption of crystalline structure of the cellulose fibrils is also evident in many chemical pretreatment methods as indicated by the fact that many pretreated biomasses has shown digestibility higher than that of pure α-cellulose [35 – 37].

The effectiveness of dilute acid hydrolysis as a pretreatment has been verified experimentally. Researchers at NREL have characterized the susceptibility of a variety of short rotation woody and herbaceous crops and agricultural residues upon dilute acid pretreatment [17, 38 – 41]. What is more interesting is that the maximum digestibility usually coincides with complete hemicellulose removal. The dilute acid treatment of biomass aimed at hemicellulose hydrolysis has since become a widely accepted pretreatment method for enzymatic hydrolysis [16, 42 – 44].

3.5 Variations of Percolation Reactor Operation

3.5.1 *Step Change of Temperature and Optimum Temperature Difference*

Further improvements in the percolation reactor performance can be obtained by exploiting the kinetics of the hydrolysis reaction. The hemicellulose has been modeled to be biphasic as stated in the kinetic section [15, 21, 45 – 47] i. e. it is composed of two different fragments (a fast-hydrolyzing fraction and a slow-hydrolyzing fraction). The biphasic nature of the substrate brings about a number of interesting points concerning the reactor design and operation, especially with regard to the temperature policy and flow configuration in the reactor system. For a simple serial reaction, high temperature is preferred because of the activation energy difference between hydrolysis and decomposition. For biphasic substrates, however, applying uniformly high temperature may cause ex-

cessive decomposition of the sugar released from the fast-hydrolyzing hemicellulose fraction, which builds up at the early phase of the reaction. Therefore, the uniform temperature policy may not be the best solution. Kim and Lee [48] verified the impact of the non-uniform optimum temperature policy on theoretical grounds.

The concept involves two-stage processing of biomass, a low-temperature stage and a high-temperature stage. They found that variation in the temperature during the process, especially a step change from uniform low to uniform high, gives better results than either limit case. They have further determined the optimum temperature difference in step-change operation to be 30 °C for a wide range of reaction temperature. The increases in yield was about 6.5 % over that of the low uniform temperature case and about 2.5 % over the uniform high temperature case for hybrid poplar. In another study involving a corn cobs/stover mixture (CCSM), increase in yields by 3–11 % (depending on the reaction conditions) over that of the uniform temperature operation has been reported [22].

3.5.2
Two-Stage Reverse-Flow Percolation Reactor

Kim et al. [49] introduced a further variation in the percolation reactor operation involving a two-stage reverse-flow scheme. The advantages of it were later verified experimentally by Torget [9]. The process is shown schematically in Fig. 4. The biomass is first treated at a low temperature in percolation mode. It is then treated again at a high temperature. Up to this point, the procedure is identical to the two-stage process with a step change in temperature. The difference is that the stream from the high-temperature treatment is again put through a reactor packed with fresh biomass at low temperature. The reacted solid residue in this reactor is then treated with fresh acid at high temperature. This process is repeated. Figure 4 also illustrates the difference between the temperature step change and the two-stage reverse-flow configuration.

The simulation results of the two-stage reverse-flow reactor arrangement with temperature change indicate that further improvements in performance over that of non-isothermal operation can be achieved. Chen et al. [22] in their modeling study found that the sugar concentration essentially doubles over that of the best-case percolation reactor employing temperature step change only. Viewing this from another angle, about 5 % improvement in product yield is obtained over that of the temperature step-change operation for the same product concentration [49]. These modified versions of the percolation reactor and their operational strategies concern mostly the modeling work. They have been suggested as a method of pretreatment for enzymatic hydrolysis.

3.6
Counter-Current Reactor

The most unusual development in reactor design in dilute acid hydrolysis of cellulose hydrolysis is that of a counter-current reactor. It is defined as a mov-

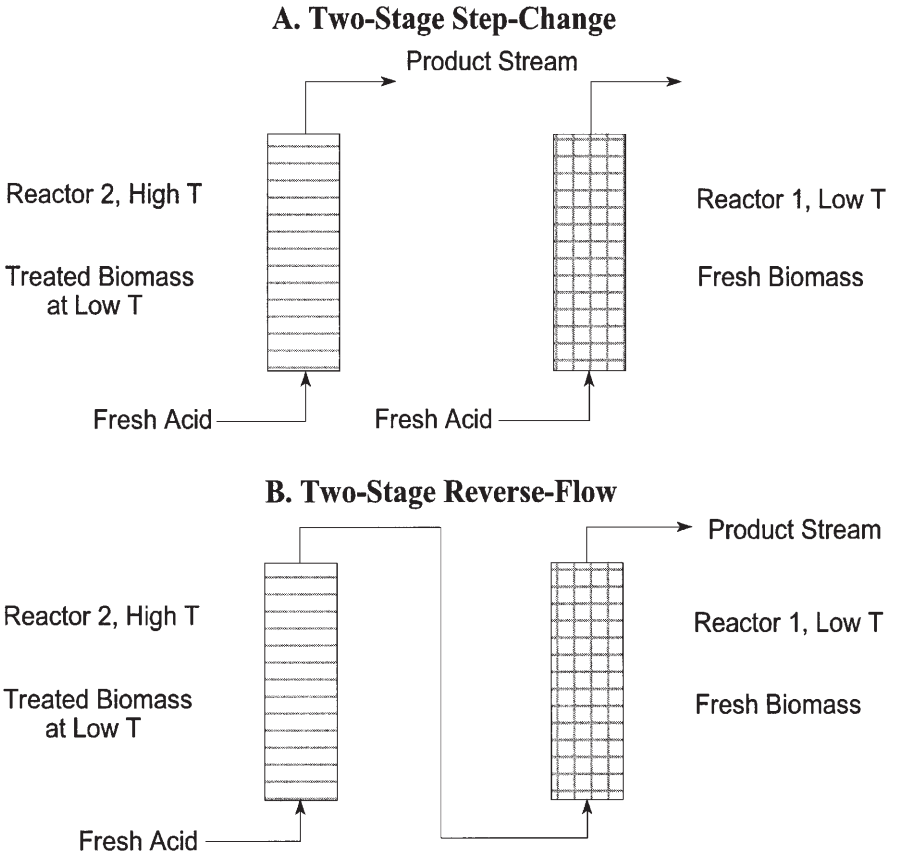


Fig. 4. Schematics of temperature step-change and two-stage reverse flow

ing-bed reactor in which the direction of solid and liquid are reversed (Fig. 2). It was first introduced and analyzed by Song and Lee [7] as a conceptual reactor. The modeling results of this reactor indicate that it outperforms all other known reactors in acid saccharification of lignocellulose rendering high yield and sugar concentration [7]. The supremacy of a counter-current reactor is best explained by the sugar profile curves shown in Fig. 5. In this figure, the calculated sugar concentration profiles are shown for a co-current and a counter-current reactor. These curves were calculated for identical reaction conditions (a representative condition for cellulose hydrolysis) except that the flow directions of solid and liquid are reversed. In the counter-current reactor scheme, the major portion of the sugar is produced in the vicinity of the liquid outlet point. The stream then travels a relatively short distance before it is washed out of the reactor, thus reducing the time period for the sugar to decompose, consequently raising the yield and sugar concentration.

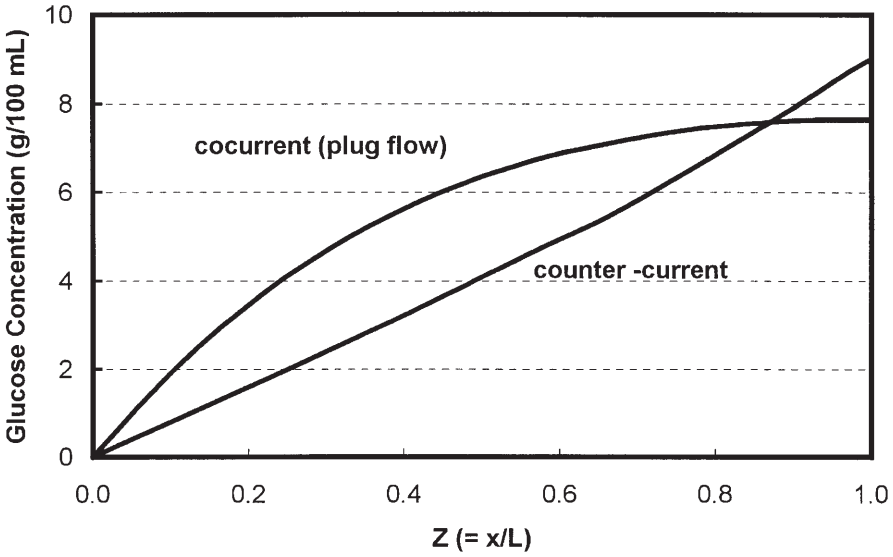


Fig. 5. Profiles of sugar concentration in co-current and counter-current reactors. Substrate: yellow poplar; reaction conditions: acid concentration = 0.08 wt% sulfuric acid, u = 1.0 cm/min, vo = 1.0 cm/min, T = 230°C, reactor length = 6 inches

3.6.1 *Progressing Batch Reactor*

Wright et al. [6] made the first attempt at the design and operation of a counter-current reactor in dilute acid hydrolysis of cellulose. In this design, a bank of percolation reactors connected in series was used in order to simulate the counter-current flow pattern. The system used seven percolation reactors in total, one reactor for filling, another for emptying, five in operation. Two of the five reactors were used for prehydrolysis (for hemicellulose), and three for main hydrolysis (cellulose). The five-reactor system was put into operation for a preset duration after which one reactor at the liquid entering side was disconnected for emptying while another reactor prefilled with biomass was added to the other side of the cascade. As this process was repeated, a counter-current flow was simulated, reactors containing biomass moving in one direction, the flow of liquid moving in the other direction. The actual operation was done with flow switching not involving movement of reactors. While a significant improvement was predicted from an ideal counter-current reactor over a single-stage percolation reactor (20% increase in yield and concentration), a number of potential problems were also cited. As the hydrolysis reaction progresses, up to 80% of the wood is converted to soluble products. The wood particles also lose their rigidity and collapse increasing the voidage from approximately 35 to 85% [6], a rather drastic departure from the model assumption. They also pointed out that mass and heat transfer effects within chip size solid particles might adversely

affect the reactor performance. This was verified in single-reactor hydrolysis experiments using wood chips of 1/8–1/4 inch [50]. The sugar concentrations obtainable from the single-reactor study were lower than those reported in previous studies [51]. Overall the performance of the progressing batch hydrolysis reactor was far below the level predicted by the ideal reactor model due to the severity of non-ideal behavior and the operational difficulties.

3.6.2 *Effect of Bed Shrinking*

A very significant portion of cellulosic biomass is converted to soluble products during acid hydrolysis. This causes either collapse of bed or increase of bed voidage. This was one of the major problems associated with operation of a percolation reactor as noted by Wright et al. [6]. This very phenomenon, although perceived here as a negative point, can become a positive point. In order for this to happen, one condition must be met, i. e. that the bulk density of the solid (or the solid/liquid ratio) in the reactor must remain relatively constant. In this regard, a unique packed reactor was designed by Torget et al. [10] as shown in Fig. 6. The reactor has a fixed and a movable end; the movable end is supported by a compressed spring. As the reaction progresses, gradual depletion of the biomass occurs, making the bed less dense. The spring-attached movable end

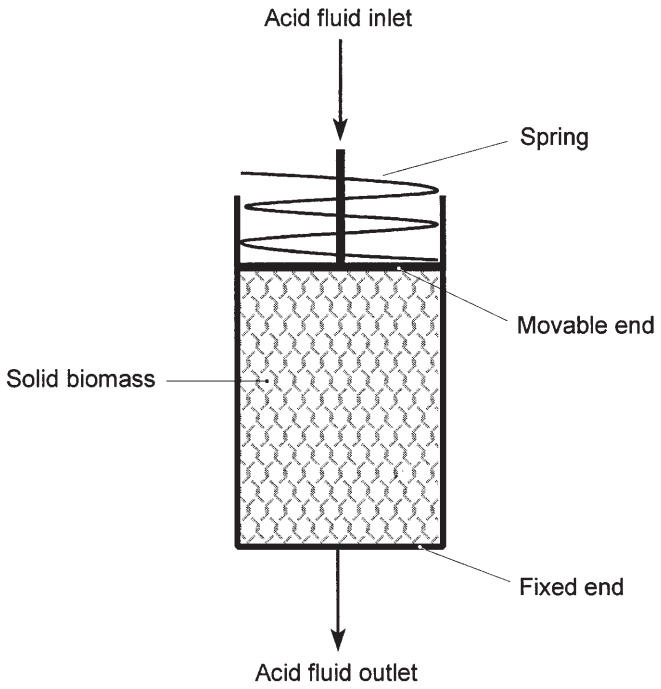


Fig. 6. A simplified diagram of a shrinking-bed reactor

then takes effect pressing the loose biomass particles maintaining the bulk packing density constant. This is strictly a proof-of-concept type reactor designed specifically to keep the bed packing density constant. This is also a reactor with a shrinking-bed depth (reactor volume), hence named a shrinking-bed reactor.

In a shrinking-bed reactor, the flow pattern of liquid becomes more consistent. We now have a well behaved packed-bed reactor throughout the operation without any dead zone. More importantly, the bed shrinkage increases the relative liquid throughput per solid mass in the reactor. We have previously introduced one of the dimensionless parameters in the percolation reactor, $\tau = ut/L$. As this parameter increases, the yield increases, but the concentration is expected to decrease (see Fig. 3). However, in the bed-shrinking reactor, we can increase this parameter simply by decreasing the bed depth (L) without increasing the liquid input. The net effect of all this is that an increase in the yield without a decrease in the concentration can be expected. Such an improvement in the reactor performance has been verified experimentally [52] as well as theoretically [53]. The experimental setup of Torget et al. (1998) is based on the shrinking-bed reverse-flow three-stage percolation reactor shown in Fig. 7. This reactor assembly is similar to that of the "progressing batch reactor" previously described [6]. This has three active reactors, one for prehydrolysis and two for main hydrolysis. It is still a simulated counter-current reactor as the direction of liquid flow is the opposite of the reactor movement. The performance of this reactor system is summarized in Fig. 8. Shown in the figure are 83% yield of glucose and near quantitative yield of xylose with 3.6% total monomeric sugar

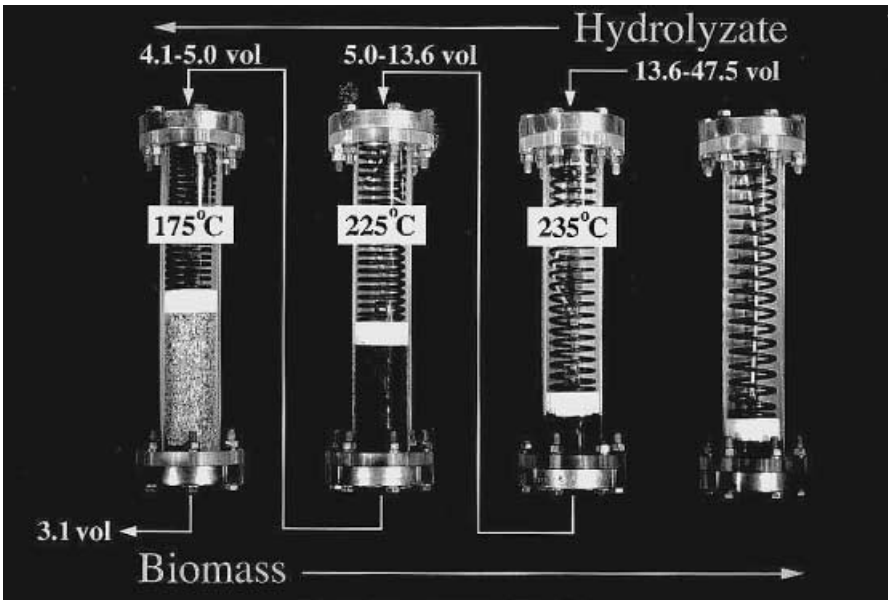


Fig. 7. Shrinking-bed reverse-flow three-stage percolation reactor

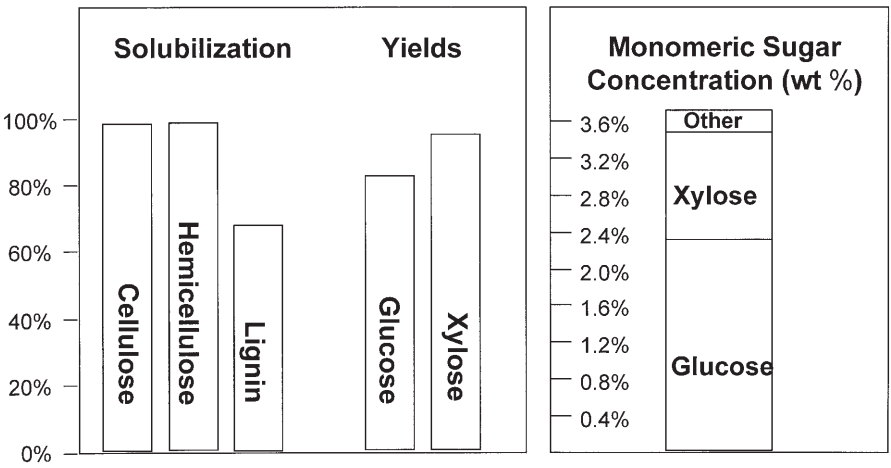


Fig. 8. Three-stage shrinking-bed reactor performance

concentration. It is indeed a phenomenal improvement over that of any other known reactor to date. It is a development significant enough to change the perspective of acid hydrolysis technology and perhaps may even emerge as a viable alternative in biomass saccharification.

3.7 Shrinking-Bed Counter-Current Reactor

The shrinking-bed counter-current reactor demonstration was limited to theoretical studies and proof-of-concept experiments dealing only with a simulated counter-current scheme. It is conceivable that a continuous counter-current reactor that retains the property of bed shrinking can bring about additional improvements in the reactor performance. A theoretical investigation was conducted by Iyer et al. [54] to study the effect of bed shrinking in a continuous counter-current reactor. It is noteworthy that the concept of shrinking bed causes the change of solid velocity within the reactor. The advantage of the shrinking-bed operation over the non-shrinking one is clearly demonstrated in the simulation results shown in Fig. 9. The thin lines connecting the yield and concentration represent the constant liquid velocity contours under shrinking- and non-shrinking-bed operation. As shown in Fig. 9, at a liquid velocity of 3 cm/min, the yield for cellulose hydrolysis is 88% under shrinking-bed operation and 69% under the non-shrinking-bed operation. It is also noted that glucose concentration is much lower in the non-shrinking mode than that in the shrinking mode under identical operating conditions. The drop in yield and concentration in the non-shrinking operation is not entirely due to sugar degradation. The extent of conversion was 99.9% under shrinking while it was only 81% under non-shrinking under identical process conditions. In other words, the optimum solid feeding rate was much lower under non-shrinking than that

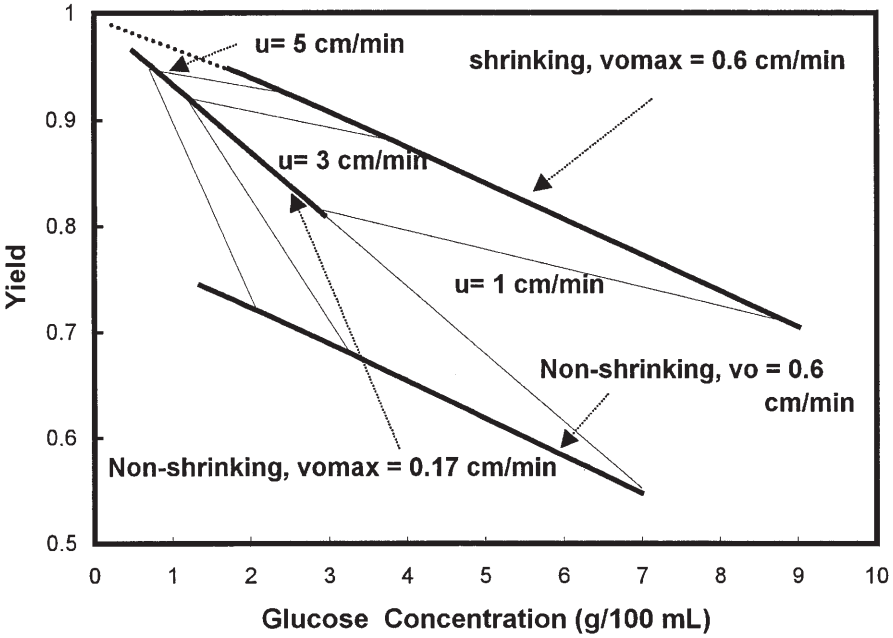


Fig. 9. Yield vs. product concentration in shrinking-bed and non-shrinking-bed counter-current flow reactor. Substrate: yellow poplar; reaction conditions: acid concentration = 0.08 wt% sulfuric acid, T = 230°C, reactor length = 6 inches

under shrinking for the same process conditions. At a liquid velocity of 3 cm/min, the maximum solid feeding rate was found to be only 0.17 cm/min under non-shrinking while it was as high as 0.6 cm/min for shrinking. Even under the reduced solid feeding rate, the yield obtained under non-shrinking is lower than that obtainable under shrinking at any given level of sugar concentration. Taking a sample point in the modeling, in order to achieve a glucose yield of 92%, the simulation results in Fig. 9 indicate that a glucose concentration in the product stream of 2.4 g/100 ml is obtained for the shrinking-bed operation. For the non-shrinking operation, however, the product concentration corresponding to the same yield is only 1.1 g/100 ml, a difference of a factor of two.

Work is currently in progress at NREL to develop a pilot-scale continuous counter-current reactor that retains the property of bed shrinking. This 200 kg/day unit is being constructed in cooperation with Sunds, Inc., Norcross, GA, USA. It consists of two screw-driven reactors (Fig. 10). The first reactor is a screw-fed horizontal plug flow reactor. It is designed for prehydrolysis in which the hemicellulose fraction of the biomass is hydrolyzed. The main hydrolysis reactor is of vertical design with solid moving upward. It is equipped with counter-current mechanism. It contains a broken flight screw within the reactor and two additional screws for solid feeding and withdrawal. The liquid is driven down the reactor by exerting a positive pressure above the reactor pressure to

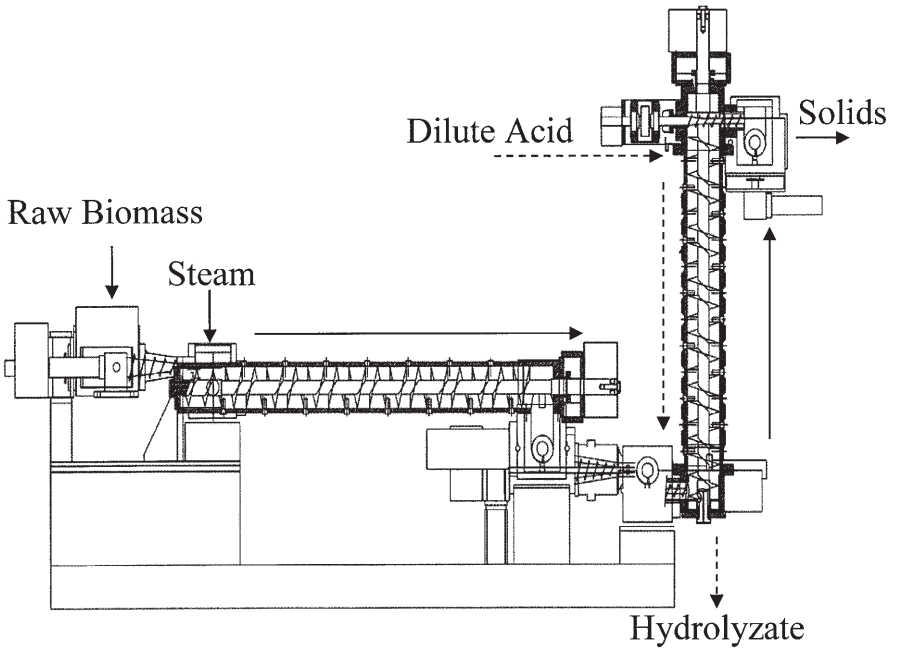


Fig. 10. Continuous counter-current shrinking-bed reactor

overcome frictional forces and forces exerted by the solids moving upward. A large part of the feasibility study on a true counter-current reactor may come from the test data from this system.

4 Economic Factors

A few economic factors concerning the dilute acid process are discussed here in comparison to the enzymatic process. It must start with the yield that has been the focus of the reactor analysis and development. Production of fuels and chemicals from biomass is substrate cost intensive with feedstock costs representing roughly one half of total production costs [42, 55]. It is of paramount importance in most bioconversion processes to maximize the yield of the product from the biomass substrates. The most advanced dilute acid process has brought the saccharification yield to above 80% as verified by NREL bench-scale experiments [10]. The actual yield of the enzyme saccharification is dependent on the effectiveness of the pretreatment. However, it rarely surpasses 90% even with a highly efficient pretreatment. The enzymatic process also requires additional substrate because it has to be produced from cellulosic biomass. The amount of biomass feedstock needed for enzyme production is estimated to be about 9% of the total. With these in consideration, the yield of the acid saccharification is about even with that of the enzymatic process. What can

make the acid process more favorable is that the total cost incurred by the enzyme including the production, operation and capital charge is as high as $0.21 per gallon of ethanol according to the NREL process economics model [56].

On the down side, the cost of energy (steam input) in operating an acid hydrolysis reactor is higher than that in an enzymatic or a simultaneous saccharification and fermentation reactor. Furthermore, xylose is obtained predominantly in oligomeric form. Further processing of the liquor at 135°C for four hours is needed to breakdown the sugars completely into monomers. Also, the hydrolysates from the acid process contain various degradation products that are toxic to microorganisms. Detoxification of the hydrolysates is therefore a necessary element in the integral acid hydrolysis process. This would be an extra cost item in comparison to the enzymatic process.

There does not seem to be a clear-cut edge on either side as far as equipment cost is concerned. The acid process requires an additional reactor that would probably be made out of high nickel material. In its defense, the enzymatic process also carries a pretreatment reactor, which is very similar in design and cost to the acid hydrolysis reactor. Also, the acid-based process simplifies the biological part of the process converting the SSF reactor into a regular fermenter.

5
Future Issues

A continuous counter-current reactor system has shown great promise as a process reactor in the dilute acid hydrolysis of cellulose. However, the findings on this unique reactor system have been limited to the theoretical aspects and the proof-of-concept laboratory experiments. It has to be developed into an upscale process reactor before it is adopted into the biomass conversion process. To this end, a pilot-scale process study is being conducted at NREL. This is only the first step. To be noted here is that reactors of similar design are being used in industry; it took years of developmental work, however, before they were put into commercial service. It would probably take about the same degree of investment in this case. In addition to the reactor issue, there are other important issues that need to be addressed in the dilute acid process.

5.1
Toxicity

The crude sugar solution from the acid process contains various degradation products. There are lignin breakdown products that are basically low molecular weight lignins, also known as acid-soluble lignin. Various sugar decomposition products also exist including furfural, (hydroxymethyl)furfural and formic and acetic acid. Most of these components are toxic to microorganisms showing negative effects on the subsequent fermentation process. This is a well-known problem associated with the acid hydrolysis process yet to be resolved. A number of different detoxification methods have been investigated. Neutralization with lime and charcoal treatment are among the methods attempted with varying degrees of success. There is no simple uniform solution available because

the problems themselves are quite diverse as they vary depending on the species of feedstock, reaction conditions and the tolerance of the microorganisms. They have to be solved on a case by case basis. The solutions may come from various angles: treatment of liquid feed, adaptation of the microorganisms to enhance the tolerance. Some novel ideas such as employing high cell density and/or using immobilized cells as a means of overcoming or protecting the cells from toxins may play a role in a scheme of the solution. At this time it is a significant cost factor for most of these methods. It remains as one of the key issues in the acid-based technology.

5.2 Utilization of Lignin

As a biomass component of highest specific heat content, lignin accounts for about 30–40% of the overall biomass heat content [57]. For most biomass feedstocks, the amount of lignin present is so high that excess electricity can actually be generated beyond that required for the process needs [56]. Additional revenue can thus be generated from upgrading of the excess lignin. In the dilute acid processing of cellulosic components, especially under high-temperature conditions, more than half of the lignin content in hardwoods (50–70%) is solubilized into liquid due to partial cracking of lignin. A substantial fraction of this lignin (often referred to as acid-soluble lignin) actually precipitates out from the sugar solution during storage at room temperature. The precipitation is believed to be the result of recondensing of low-MW lignins. It is a clean feedstock very much amenable for further conversion. Research along these lines is currently in progress at NREL. Among the early findings is that the reprecipitated lignins have molecular weights much lower than Kraft lignin (about one-fourth on average). They are in general more reactive in aqueous or solvent phase treatment. During the initial phase of the cracking process the lignin undergoes a partial breakdown into smaller lignin molecules before they are converted into other organic substances [58]. One can view this lignin as a partially processed Kraft lignin readily convertible to fuels and chemicals.

As a complex amorphous phenolic polymer, lignin can be broken down into phenolic compounds and hydrocarbons [59]. The phenolic fraction can be reacted with alcohols and converted to methyl or ethyl aryl ethers (MAEs or EAEs) which are oxygenated ethers similar to methyl *tert*-butyl ether. MAEs have already been fleet tested as octane enhancers that are fully compatible with gasoline [60]. The lignin-derived fuel additive has another advantage in that it has a lower vapor pressure than methanol, ethanol, or the ether octane enhancers currently being used. Lignin can also be converted into a variety of chemical products including phenolic compounds, aromatics, dibasic acids, and olefins [61]. The yield of reprecipitated lignin in acid hydrolysis is 5–20% of dry biomass feed. Upgrading of this material into a marketable by-product would be an important issue that could bring about a significant impact in the overall process economics.

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Genetic Engineering for Improved Xylose Fermentation by Yeasts

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Xylose utilization is essential for the efficient conversion of lignocellulosic materials to fuels and chemicals. A few yeasts are known to ferment xylose directly to ethanol. However, the rates and yields need to be improved for commercialization. Xylose utilization is repressed by glucose which is usually present in lignocellulosic hydrolysates, so glucose regulation should be altered in order to maximize xylose conversion. Xylose utilization also requires low amounts of oxygen for optimal production. Respiration can reduce ethanol yields, so the role of oxygen must be better understood and respiration must be reduced in order to improve ethanol production. This paper reviews the central pathways for glucose and xylose metabolism, the principal respiratory pathways, the factors determining partitioning of pyruvate between respiration and fermentation, the known genetic mechanisms for glucose and oxygen regulation, and progress to date in improving xylose fermentations by yeasts.

Keywords. Xylose, Fermentation, Respiration, Metabolic engineering, Regulation, Regulatory mechanisms, Pentose metabolism, Oxygen regulation, Glucose regulation

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1 Introduction

Commercial bioconversion of lignocellulose to ethanol requires the efficient fermentation of sugar mixtures [1, 2], otherwise product yields are low and waste disposal costs are excessive. Lignocellulose contains five major sugars, the abundance of which varies with the feedstock [3]. They are the hexoses, D-glucose, D-mannose, and D-galactose, and the pentoses, D-xylose and L-arabinose. Fructose and sucrose are not normally found in lignocellulose. The fermentation of glucose by *Saccharomyces cerevisiae* has been established through thousands of years of practice. In contrast, the objective of producing ethanol from pentose sugars has arisen relatively recently and, despite much effort in several laboratories around the world, it remains problematic.

Bacteria will ferment pentoses to ethanol plus mixed acids [4], and various researchers have genetically engineered bacteria to improve ethanol production from pentoses. However, most industrial ethanol fermentations use *S. cerevi-*

siae, and many industrial fermentations of pentose-containing wood sugars have identified or investigated yeasts for xylose utilization [5, 6]. So even though many bacterial routes exist, the fermentation of pentoses by yeasts remains of interest. The objective of this chapter is to review the metabolic regulation of ethanol production from D-xylose and L-arabinose by yeasts. The following convention for genes, proteins and mutants has been used. Acronyms for proteins are shown with the first letter capitalized. For example, alcohol dehydogenase 1 = Adh1. The gene coding for that protein is shown in italicized capitals (*ADH1*). Mutants of the gene are shown in lowercase italics (*adh1*).

1.1 Significance of Xylose in Bioconversion of Lignocellulose

About 45% of the total dry weight of wood is cellulose, the hydrolysis of which yields glucose. In agricultural residues, cellulose comprises 8 to 35% of the total dry weight. Glucose is also present in hemicellulosic sugars. Overall, it averages about 31% of the total dry weight in these materials [7]. The prevalence of glucose in starch and other storage carbohydrates such as sucrose makes D-glucose the most abundant carbohydrate in terrestrial plants. Xylose is the second most abundant sugar and it is especially prevalent in angiosperms (flowering plants). In woody angiosperms (hardwoods), D-xylose averages about 17% of the total dry weight (Fig. 1), but in herbaceous angiosperms (such as residues from agricultural crops) it can range up to 31%. Other sugars such as mannose, galactose

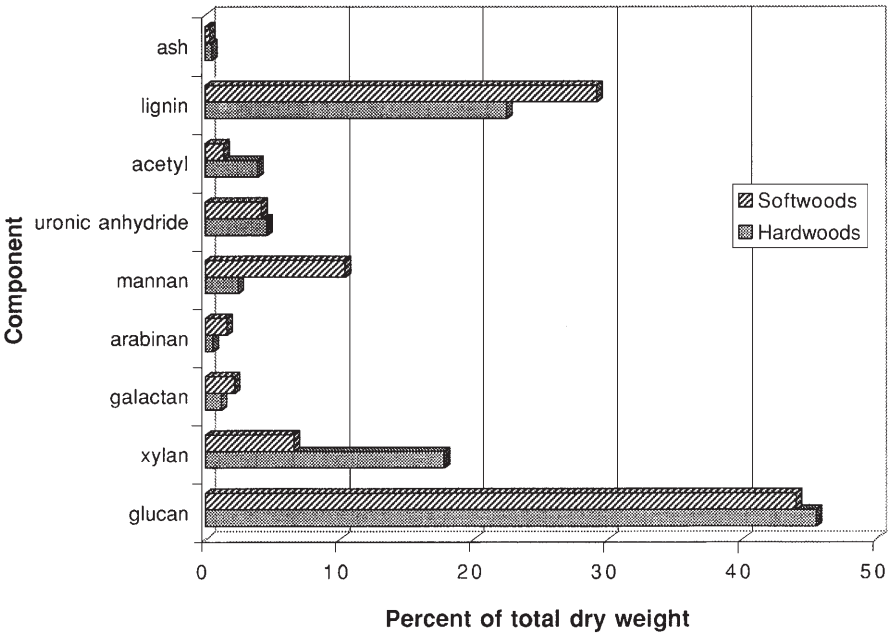

Fig. 1. Average composition of hardwood and softwood species found in North America. Adapted from [3]

and arabinose are found in the glucomannan, arabinoxylan and glucuronoxylan hemicellulosic components. Glucomannan is the main hemicellulose of gymnosperms (softwoods). In these plants, mannose comprises about 10% of the total dry weight. The lignin content is slightly higher in softwoods than in hardwoods, and because the lignin is more cross-linked in softwoods, it is harder to remove. Although the glucose and mannose present in softwoods can be fermented readily, timber and pulp manufacture places high values on the straight trunks and long fibers. Therefore, agricultural residues and fast-growing hardwood species are most commonly considered for fuel ethanol production. The high content of xylose in these materials requires that it be used efficiently.

1.2 Xylose Fermentation by Fungi and Bacteria

Early studies noted that yeasts and other fungi do not ferment pentoses but that certain bacteria can (see, for example, [8]). One of the earliest reports that fungi could convert xylose to ethanol came in 1922. Willaman and co-workers showed that the plant pathogen *Fusarium lini* could ferment xylose and accumulate ethanol to concentrations as high as 3.5% [9]. In 1935, Plevako and Cheban reported that *Monilia murmanica* and *Oidium laminarium* produced small amounts of ethanol from xylose, but under aerobic conditions they mostly produced cell mass [10]. *S. cerevisiae* does not ferment xylose, but Dickens reported in 1938 that homogenates of yeast readily fermented pentose D-ribose-5-phosphate to ethanol and could convert D-xylose-5-phosphate and L-arabinose-5-phosphate to lesser extents [11]. This suggested that the biochemical mechanisms for fermenting pentose phosphates exist in *S. cerevisiae* even if the assimilative enzymes do not. By 1945, various fusaria had been used to ferment sulfite waste liquors and hydrolysates [12]. Karczewska reported in 1959 that *Candida tropicalis* would directly convert D-xylose and L-arabinose to ethanol under low aeration conditions [13] and that *C. tropicalis* and *Candida krusei* would ferment sulfite waste liquors [14]. She also noted that several other Russian and Central European researchers had reported conversion of xylose to ethanol by various yeasts and fungi, including *Hansenula, Debaromyces* and *Schwanniomyces*. Interest in pentose fermentations declined in the West after World War II but with the oil crisis in the late 1970s biomass conversion was resurgent, and the importance of xylose was again recognized. Wang, Schopsis and Schneider reported in 1980 that if xylose were converted to xylulose, several yeasts, including *S. cerevisiae, Kluyveromyces lactis* and *Schizosaccharomyces pombe*, could ferment it directly to ethanol [15]. This was considered commercially attractive because xylose could be converted to xylulose using commercial xylose (glucose) isomerase. Various yeasts could then ferment it [16–19]. Following the report of yeast xylulose fermentation, several researchers re-discovered or re-examined the direct conversion of xylose to ethanol by various fungi. These included the yeasts *Pachysolen tannophilus* [20, 21], *C. tropicalis* [22], *Candida* sp. [23], *Candida shehatae* [24] and *Pichia stipitis* [25], and the molds *Fusarium oxysporum* [26–28], *Mucor* [29, 30] *Rhizopus javanicus, Rhizopus oryzae* and several species of *Aspergillus* [31].

Bacteria convert D-xylose directly to D-xylulose with xylose isomerase, whereas yeasts and other fungi first reduce xylose to xylitol with NADPH coupled xylose (aldose) reductase (Xor). They then oxidize xylitol to D-xylulose with NAD^+ coupled xylitol dehydrogenase (Xid) [32]. This difference between bacteria and fungi might have evolved to accommodate the higher efficiency of xylose metabolism achieved through respiration. The equilibrium attained by xylose isomerase between xylose and xylulose is 77:23 at 68 °C [33] From this value, one can calculate that about + 0.82 kcal of energy is required per mole to convert xylose into xylulose. By providing NADPH through the oxidative pentose phosphate pathway and by respiring $NADH^+$, yeasts and fungi can drive the coupled redox assimilation of xylose under aerobic conditions. This reaction ties eukaryotic metabolism of xylose to respiration.

1.3 Metabolic Engineering for Improved Fermentation

Initially, the basis for xylose fermentation in yeasts and fungi was not well understood. Researchers knew that bacteria with xylose isomerase could ferment xylose while fungi with the oxidoreductase uptake system could not, so they sought to express xylose isomerase in *S. cerevisiae* or other yeasts in order to create an improved xylose fermenter. Ueng et al. [34] cloned the gene for xylose isomerase from *E. coli* and Chan et al. [35] expressed it in *S. pombe*. These are the only researchers to have reported success with this approach. Amore et al. [36] expressed the genes from *Bacillus* and *Actinoplanes* in *S. cerevisiae*. Approximately 5% of the cellular protein consisted of xylose isomerase, but it was not catalytically active. Sarthy et al. [37] expressed *E. coli* xylose isomerase in *S. cerevisiae* but found that the protein had only about 10^{-3} as much activity as the native protein from *E. coli*.

Over the past 30 years, a few researchers have reported the presence of xylose isomerase in a number of yeasts and fungi capable of rapid xylose metabolism. Because of difficulties in using genetically engineered *Saccharomyces*, Freer et al. [38] re-examined *Rhodosporidium toruloides* to see if they could confirm an earlier report that this yeast produces xylose isomerase. They reasoned that the heterologous expression of an eukaryotic enzyme could facilitate genetic engineering of xylose metabolism in *S. cerevisiae*. Unfortunately, they found that *R. toruloides* uses an oxidoreductase system like other eukaryotes. Other approaches, however, have been more successful.

Metabolic engineering [39, 40] has been used to impart the capacity for ethanol production and xylose fermentation in *E. coli* [41–45], *Klebsiella oxytoca* [46, 47], *Zymomonas mobilis* [48, 49] and *S. cerevisiae* [50–53]. In general, attempts at metabolic engineering have been more successful in bacteria than in yeasts. Although the reasons are not entirely clear, the smaller genomes and fewer feedback regulatory factors found in bacteria make these organisms much easier to work with.

Direct approaches using naturally occurring organisms have also been useful. Many yeasts and fungi will assimilate xylose to form cell mass under aerobic conditions, but it is converted to ethanol by only a few. The best yeast strains

known for fermenting xylose are *P. stipitis* and *C. shehatae.* The yeast *P. stipitis* is capable of converting xylose and all of the other major wood sugars to ethanol. Some strains even ferment xylan and metabolize lignin-related compounds [54]. Ethanol yields range between 0.3 and 0.44 g/g of substrate. Volumetric production rates are about 0.5 g/l-h [55]. Although this is suitable for some waste streams, commercial fermentation of hydrolysates specifically for ethanol production requires higher performance. *P. stipitis* and *C. shehatae* also ferment glucose, but glucose also represses the utilization of xylose, so fermentation of glucose and xylose in mixtures presents a complex challenge. Because of the rarity of xylose-fermenting yeasts, it is appropriate to ask what makes them special. How do they take up xylose, make ethanol, respire, and coordinate metabolism? Once we better understand these processes, we can hope to modify them for improved performance.

Some of the subjects relevant to this review have been covered more extensively elsewhere. Jeffries and Kurtzman have reviewed strain selection, taxonomy and genetics of xylose-fermenting yeasts [56], Hahn-Hägerdal et al. [57] have reviewed physiology and Du Preez [58] has reviewed fermentative processes. Lee has reviewed the structure and function of yeast aldose reductases [59] and Boles and Hollenberg have reviewed hexose transport [60]. Alexander and Jeffries have reviewed regulatory aspects of metabolite partitioning [61]. Gonçalves et al. [62] have reviewed the signaling pathways for the regulation of glycolysis and Kwast et al. have reviewed the regulatory mechanisms imparted by oxygen [63]. The present review will attempt to integrate these topics with genetic aspects of the yeast D-xylose fermentation.

2
Glucose and Xylose Transport

Sugar transport is mediated by two different systems: carrier mediated facilitated diffusion, and active proton symport. Facilitated diffusion is energy independent and functions well at elevated sugar concentrations. Proton symport requires energy but is useful during growth at low extracellular sugar concentrations. In *S. cerevisiae*, the transport of glucose into the cells plays a direct role in sensing glucose and in signal transduction. *S. cerevisiae* uses facilitated diffusion systems to take up hexoses but uses proton symport systems to take up disaccharides. *S. cerevisiae* can handle wide ranges of sugar concentrations up to 1.5 M by developing a group of Hxtp proteins. The presence and the concentration of the substrates tightly regulate expression of these proteins.

In *S. cerevisiae,* hexose uptake is mediated by a large number of related transporter proteins. Six out of 20 genes for hexose transport mediate the uptake of glucose, fructose and mannose at metabolically relevant rates. Two others catalyze the transport of only small amounts of these sugars. One protein is a galactose transporter but is also able to transport glucose. Hexose transport (*HXT*) deficient mutants have no clearly detectable phenotypes. Expression of *HXT1, 2, 3, 4, 6* or *7* is sufficient to allow various degrees of glucose utilization [64]. Cells with *HXT1–7* deleted do not grow on any concentration of glucose,

fructose or mannose [65, 66]. Both low- and high-affinity glucose uptakes are totally abolished in *hxt1–7* mutants [67].

Two transport-related proteins, Snf3 and Rgt2, act as glucose sensors in a signal transduction pathway through Grr1 [68]. The Snf3 high-affinity glucose transporter is required to induce several hexose transporters by low levels of glucose. Rgt2 is required for maximal induction by high levels of glucose. The Grr1 pathway triggers a signal in response to the level and availability of glucose present in the medium [69, 70].

In yeasts that utilize both xylose and glucose, these sugars share the same transporter systems [60]. Glucose can inhibit the xylose uptake by competing with the xylose transporters. Even 0.05 mM glucose can compete with xylose uptake, which significantly reduces xylose transport. Xylose transport in *P. stipitis* is mediated by low- and high-affinity proton symporters [71]. Both transporters are constitutively expressed with low V_{max} values. The low-affinity system takes up glucose in the range of 0.3 to 1 mM. Moreover, inhibitor studies indicate that uptake of xylose requires aerobic respiration, which suggests that both systems involve proton symport [72].

A putative xylose transporter gene from *P. stipitis*, *PsSTU1*, has been cloned recently. This gene can confer high-affinity uptake of glucose and growth to a *S. cerevisiae hxt1–7* strain [60]. The deduced amino acid sequence shows 54% identity to the HXT glucose transporters of *S. cerevisiae*. When *PsSTU1* is introduced into *S. cerevisiae*, it can transport xylose but with a considerably lower affinity than what is achieved in *P. stipitis* [71]. This suggests that the transport system in *P. stipitis* is coupled to other factors. Two other *STU*-related genes have been identified from *P. stipitis* by cross-hybridization with *S. cerevisiae* transporter genes as probes. Uptake of xylose in *S. cerevisiae* is mediated nonspecifically and with low affinity by the hexose transporters, so other factors would be required to increase xylose metabolism in this yeast.

In *S. cerevisiae* genetically engineered for xylose uptake, glucose, mannose, and fructose inhibited xylose conversion by 99, 77, and 78%, respectively. These sugars are transported by the same high affinity transport system as xylose and the results are thought to reflect competitive inhibition of xylose transport [73]. Galactose is less inhibitory to xylose transport than glucose and was therefore a better cometabolizable carbon source for xylitol production.

3 Glycolysis

Figure 2 outlines the metabolism of glucose and xylose. The two pathways merge at the level of fructose-6-P and glyceradehyde-3-P. They share all of the energy-producing substrate-level reactions of glycolysis and, on a carbon basis, the ATP required for activation is the same for xylose and glucose metabolism. By the reaction pathway shown, six molecules of xylose require six ATP to form four fructose 6-phosphates and two glyceraldehyde 3-phosphates. Metabolism of five glucose moieties requires six ATP to form the same products. However, the two sugars differ dramatically in the steps leading up to these intermediates. Glucose is phosphorylated directly whereas xylose must first go through a re-

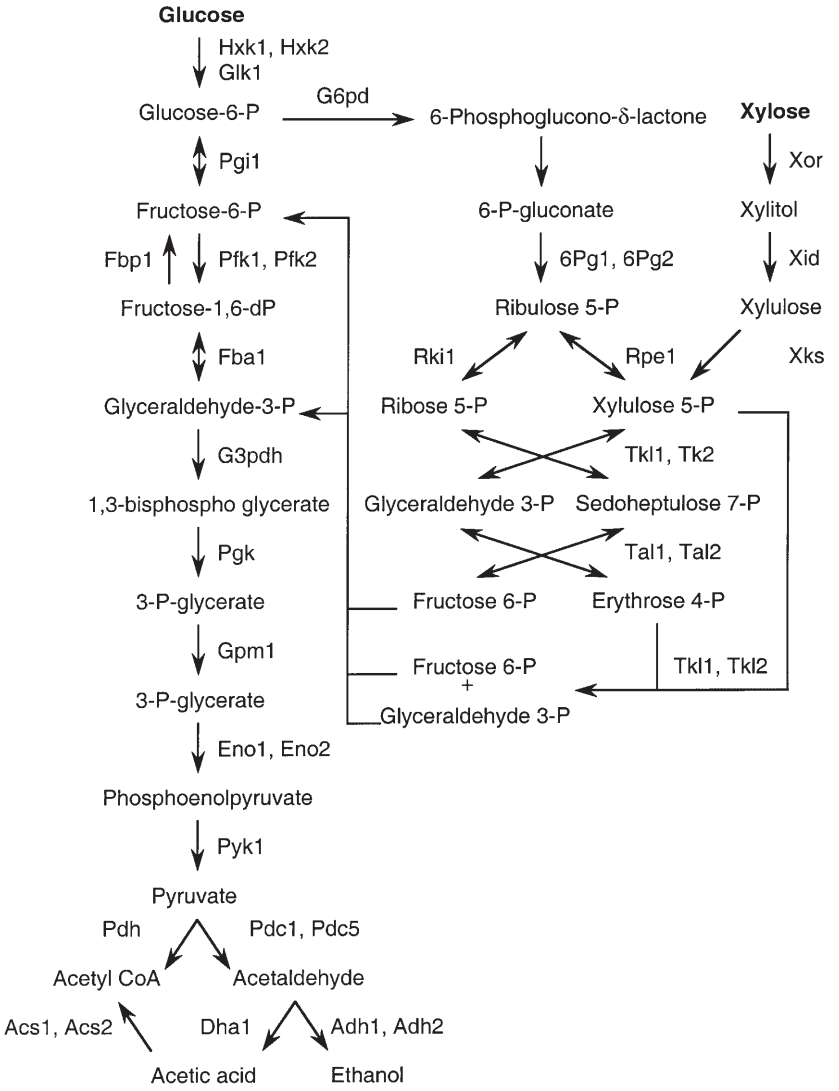

Fig. 2. Outline of glucose and xylose metabolism in yeasts. Enzyme designations are from assigned loci in *Saccharomyces cerevisiae* or *Pichia stipitis*. *Hxk1* Hexokinase P1; *Hxk2* hexokinase PII; *Glk* glucokinase; *Pgi* phosphoglucose isomerase; *Pfk* phosphofructokinase 1; *Fba* fructose-bisphosphate aldolase; *Tdh* (G3p) glyceraldehyde-3-phosphate dehydrogenase; *Pgk* 3-phosphoglycerate kinase; *Gpm* phosphoglycerate mutase; *Eno* enolase (2-phosphoglycerate dehydratase); *Pyk* pyruvate kinase; *Pdc* pyruvate decarboxylase; *Adh* alcohol dehydrogenase; *Pdh* pyruvate dehydrogenase; *Dha* aldehyde dehydrogenase; *Acs* acety l-coenzyme A synthetase; *6Pg* 6-phosphogluconate dehydrogenase, decarboxylating; *Rpe* ribulose-phosphate 3-epimerase; *Rki* ribose-5-phosphate isomerase; *Tkl* transketolase; *Tal* transaldolase; *Xor* xylose (aldose) reductase; *Xid* xylitol dehydrogenase; *Xks* xylulokinase

duction and oxidation. As shown in Fig. 3, hexokinase 1 (Hxk1), hexokinase PII (Hxk2) or glucokinase (Glk1) can phosphorylate glucose. Hxk1 or Hxk2 can phosphorylate fructose.

Glucose usually accompanies xylose in lignocellulose hydrolysates, and glucose represses use of other carbon sources. Because significant lag phases occur in shifting from one carbon source to another during the utilization of sugar mixtures, researchers have sought to understand and alleviate glucose repression. Addition of glucose (or fructose) to *S. cerevisiae* when it is growing on some other carbon source has short- and long-term effects. It first inactivates gluconeogenic genes and high-affinity glucose transporters, represses the synthesis of respiratory and gluconeogenic enzymes and induces the expression of various glycolytic enzymes. This rapid response is mediated by the RAS-cAMP signaling pathway, which is involved in nutrient sensing and growth regulation. Activation of this pathway depends on sugar phosphorylation. This triggers a rapid, transient accumulation of cAMP, which activates cAMP-dependent kinase (cAPK) which in turn activates or inactivates various enzymes and genes. Both *HXK1* and *GLK1* are themselves repressed by glucose or fructose, which leaves only the *HXK2* to maintain the repressed state. Any one of the three kinases can mediate a short-term response to glucose addition in *S. cerevisiae*, but the long-term response requires Hxk2 [74, 75].

Hxk2 is a phosphoprotein that exists in a mono- or a dimeric state (Fig. 4). Its degree of phosphorylation depends on the nature of the carbon source. With poor carbon sources it is phosphorylated, and it exists as a monomer. Following the addition of glucose, it is dephosphorylated, and it exists as the dimer [76]. Hxk2 is found in both the cytosol and the nucleus, which suggests that it might have a dual role in hexose assimilation and transcriptional regulation [77].

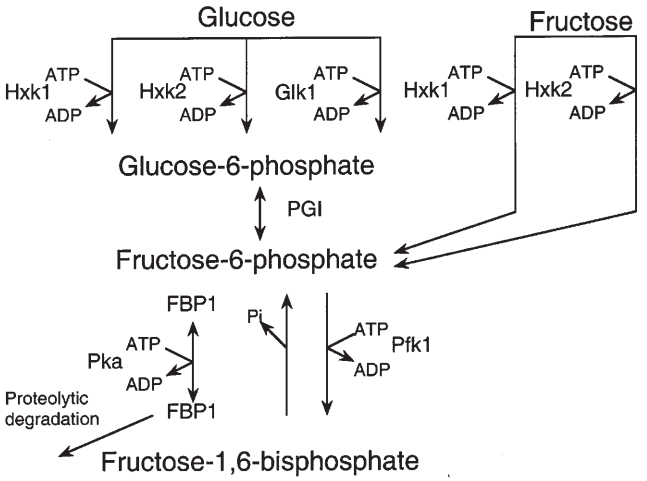

Fig. 3. Phosphorylation of glucose and fructose. *Hxk1* Hexokinase P1; *Hxk2* hexokinase PII; *Glk* glucokinase; *Pgi* phosphoglucose isomerase; *Pfk* phosphofructokinase; *Fbp* fructose-1,6-bisphosphatase; *Pka* protein kinase A

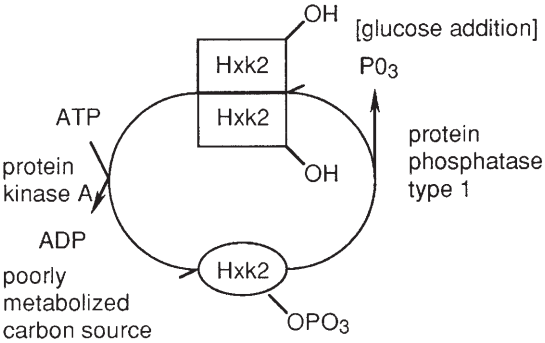

Fig. 4. Dimerization and phosphorylation of Hxk2. Hexokinase PII (*Hxk2*) was one of the first proteins to be implicated in glucose repression and adaptation to fermentative metabolism. However, its role is still unclear. Hxk2 is a phosphoprotein which exists in a mono- or dimeric state. Only the monomer is phosphorylated. Hxk2 is found in the cytoplasm and the nucleus which suggests that it may play roles in both hexose metabolism and transcriptional regulation [76, 77]

4 Pentose Metabolism

Pentose metabolism is tied to hexose metabolism through the oxidative pentose phosphate shunt. Glucose-6-phosphate dehydrogenase (G6Pd) catalyzes the first step in the oxidative pentose phosphate pathway. Dehydrogenation and hydration of glucose-6-P to form 6-P-gluconate is essentially irreversible, and the activity of G6Pd is closely regulated by the intracellular concentration of $NADP^+$. The hydration of 6-P-glucono-Δ–lactone is one of the few reactions in cellular metabolism that proceeds without a catalyst. The decarboxylation of 6-P-gluconate by 6-P-gluconate dehydrogenase (6Pg) to form ribulose-5-P likewise produces NADPH. Together, these two reactions provide most of the reductant necessary for anabolic metabolism, and other reactions such as sulfate and nitrate reduction. In *S. cerevisiae*, G6Pd is coded for by *ZWF1* [78]. The expression of *ZWF1* does not appear to be transcriptionally regulated. The reaction mediated by G6Pd is essentially irreversible, and the intracellular level of $NADP^+$ regulates its activity. Formation of $NADP^+$ is favored. The ratio of $NADP^+$ to NADPH in rat liver is about 0.014. By comparison, the ratio of NAD^+ to NADH in the same tissue is 700 [79]. In most yeasts and fungi, G6pd and 6PG also provide abundant reductant for the assimilation of xylose to xylitol.

Xylitol is the probable connecting point between the D-xylose and L-arabinose metabolic pathways (Fig. 5). L-arabinose is the form found most abundantly in nature. Early work by Chaing and Knight showed that cell-free extracts of *Penicillium chrysogenum* convert L-arabinose to both L-ribose and L-xylulose through the intermediate, L-arabinitol (= L-arabitol) [80]. Only one enzyme, aldose reductase, appears to be responsible for the conversion of L-arabinose to L-arabinitol. Aldose reductase also acts on D-arabinose to produce D-arabitol. Witterveen et al. obtained a mutant of *Aspergillus niger* deficient in

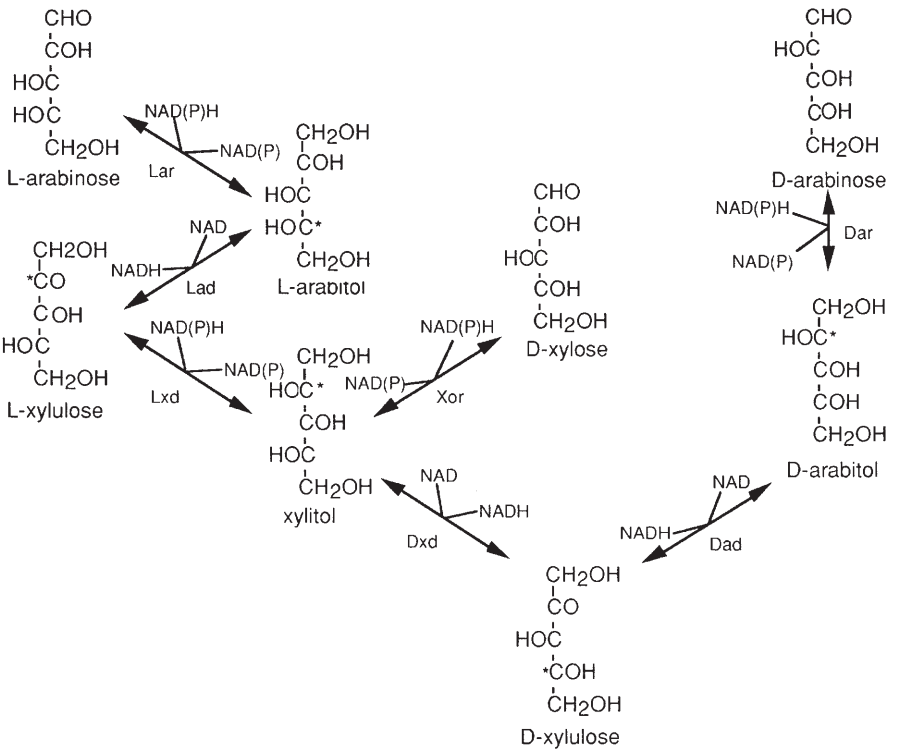

Fig. 5. Assimilation of D-xylose, L-arabinose and D-arabinose. In yeasts and fungi, pentoses are assimilated by way of oxidoreductases. D-xylose. L-arabinose and D-arabinose are each reduced to their respective polyols by aldose reductases, designated here as Xor, Lar and Dar. Both D-xylose and L-xylose are reduced to xylitol, which is symmetrical. D-xylose and L-arabinose are the forms normally found in nature. D- and L-arabitol dehydrogenases (Dad and Lad) form D- and L-xylulose, respectively. D- and L-Xylitol dehydrogenase (Dxd and Lxd) mediate the formation of D- and L-xylulose from xylitol

L-arabinose and D-xylose metabolism and determined that the lesion in this instance was in D-xylulokinase (Xks) [81]. Cultivation of *A. niger* on L-arabinose-induced NADPH-linked L-arabinose reductase (Lar), NAD^+-linked L-arabitol dehydrogenase (Lad), NADPH-linked L-xylulose reductase (Xks), NAD^+-linked xylitol dehydrogenase (Dxd) and D-xylulokinase. Witterveen et al. also demonstrated that *A. niger* forms two different pentose reductases when grown on D-xylose and L-arabinose. Both are coupled to NADPH. One, which is induced on xylose, has a broad specificity and will act on D-xylose, L-arabinose and D-ribose with equal facility. The other, which is induced on L-arabinose, shows relatively high activity for L-arabinose and significantly lower activity for D-xylose and D-ribose. De Vries et al. isolated and characterized a mutant of *Aspergillus nidulans* deficient in L-arabitol dehydrogenase (NADH) [82]. This enzyme mediates the interconversion of L-arabitol and L-xylulose, and its loss led to the accumulation of arabitol in the mutant. Witterveen et al. further iso-

lated and characterized two xylitol dehydrogenases from *A. niger* [83]. NADPH-dependent L-xylulose reductase (Lxd) converts L-xylulose to xylitol, and NAD^+-dependent xylitol dehydrogenase (Dxd) converts xylitol to D-xylulose. The stereochemistries of these reactions are shown in Fig. 5.

4.1 Xylose Reductase

Xylose (aldose) reductase (Xor) catalyzes the reduction of xylose to xylitol, which is the first step for xylose metabolism in yeasts. Most commonly, Xor is specific for NADPH. However, the main Xor of *P. stipitis* has significant activity with NADH as a cofactor [84]. Its NADH/NADPH activity ratio is 0.7. A second cryptic gene in *P. stipitis* is NADPH-dependent [85]. NADH activity is critical to the anaerobic metabolism of xylose because the second step in the pathway, xylitol dehydrogenase (Xid), is specific for NADH, and cofactor imbalance can occur unless some means exists to regenerate NAD^+ [86]. Most known Xor enzymes favor NADPH over NADH. However, in one xylitol producing yeast, *Candida boidinii*, the NADH/NADPH activity ratio of aldose reductase activity has been reported to range between 2.0 and 5.9 depending on the aeration rate [87]. When two separate aldose reductases with different NAD(P)H activities are induced to different extents, the ratio can vary. The lower ratio was observed at higher aeration rates, which is consistent with the higher respiratory function of NADPH-linked enzymes under those conditions. Xor is generally induced by xylose. Induction studies of the gene for Xor (XYL1) have been previously reviewed [55, 57].

The molecular basis for coenzyme specificity and substrate binding has been examined by several groups. In an early study, the basis for cofactor specificity was attributed to the presence of cysteine and histidine residues [88], and chemical modification studies suggested that a cysteine residue might be involved in binding of NADPH to the *P. stipitis* Xor [89]. Zhang and Lee [90] therefore mutagenized three cysteine residues in Xor to serine to determine which was responsible. The variants retained function but showed reduced catalytic activities. The apparent K_m values for NADPH, NADH and xylose did not differ by more than fourfold in the mutant enzymes. Sensitivity of Xor to thiol-specific reagents was attributed to Cys27 and Cys130 because changing these residues to serine reduced sensitivity to *p*-chloromercuriphenylsulfonic acid (pCMBS). These results suggested that these Cys residues are not directly involved in NADPH binding. Chemical inactivation studies pointed to other critical residues. Rawat and Rao [91] showed that *N*-bromosuccinimide, a reagent specific for tryptophan residues, inactivated the *Neurospora crassa* Xor and that the enzyme could be protected from inactivation by NADPH but not by xylose or NADP. This suggested that tryptophan was in the region of the NADPH binding site and that NADPH is bound more tightly than NADP. A follow-up study by the same authors showed that Cys residues involved in the reaction with *p*-hydroxymercuribenzoate (SHI) and *o*-phthalaldehyde (SHII) are distinctly different. One cysteine site (SHI) is located in a hydrophobic microenvironment at the high affinity NADPH binding site. It plays a role in the binding of the coen-

zyme to Xor, whereas a second cysteine site (SHII) maintains the conformation of the active site essential for catalysis by interacting with the NH_2 group of an essential lysine residue [92].

Other studies have shown that a conserved Ile-Pro-Lys-Ser (IPKS) [93] motif in aldose and aldehyde reductases may be responsible for the binding of NADPH [94]. The Lys230 residue has been implicated in binding to the 2′-phosphate group of NADPH in the human aldose reductase and it is conserved in yeast and human aldose reductases. Therefore, Kostrzynska et al. [95] mutated Lys270 to Met in order to determine the effect on cofactor specificity. This resulted in an 80 to 90 % reduction of the Xor activity with xylose as the substrate. The affinity of the mutant enzyme towards NADPH decreased 5- to 16-fold depending on the substrate employed. The NADH-linked activity, however, remained unchanged with glyceraldehyde as the substrate. The resulting enzyme also showed 4.3-fold higher activity with NADH than with NADPH when glyceraldehyde was the substrate. These results suggest that Lys270 is involved in both NADPH and D-xylose binding in Xor [96–98]. Comparisons with *XYL1* genes cloned from other yeasts indicate that all the known enzymes – including the *P. stipitis XYL1* – have the IPKS sequence for NADPH binding. Future research may reveal whether the Lys270Met mutation will affect NADH binding. It is still not clear why *Xor* can bind NADH in addition to NADPH or how the enzyme might be further improved for xylose fermentation by altering cofactor specificity. Results from these studies can help the understanding of the enzymatic kinetics and will enable improved engineering of *XYL1* for ethanol production.

Various research groups have independently cloned the *XYL1* gene from *P. stipitis* [99–101], *C. tropicalis* [96], *K. lactis* [97], *P. tannophilus* [98] and *C. guilliermondii* [102]; and an NADPH-specific aldose reductase [103] was identified in *S. cerevisiae* as part of its genome sequencing project. The *P. stipitis* enzyme has been overexpressed in *S. cerevisiae* [50–52, 104], and *P. stipitis* [105] in attempts to improve xylose utilization. As discussed below, the effect of *XYL1* expression depends on the genetic context. In the absence of *XYL1* and *XYL2*, *S. cerevisiae* will not grow on xylose and, in the absence of *XYL1*, *XYL2* and *XYL3*, it will not ferment. In contrast, overexpression of *XYL1* in *P. stipitis* where there is abundant *XYL2* and *XYL3* does not greatly increase fermentative activity.

Other aldose reductases have been studied. The derived amino acid sequence of the *XYL1* gene from *C. guilliermondii* [102] is only 70.4% homologous to that of *P. stipitis*. The GXGXXG motif (where X stands for any amino acid) which is responsible for the binding of the ADP moiety of NAD^+ [106] is not present in the *C. guilliermondii* or the *P. stipitis* enzyme. The authors found that when the *C. guilliermondii* enzyme was heterologously expressed in *P. pastoris*, the secreted enzyme used only NADPH as a cofactor whereas the intracellular enzyme used both NADH and NADPH. The observed ratio of NADH/NADPH activity is much lower in *C. guilliermondii* than in *P. stipitis*. In contrast, the cloned XYL1 from *P. tannophilus* exhibits significant sequence identity with other oxidoreductases [98], but evidence suggests that at least two genes for Xor are present in this organism.

The overexpression of *PsXYL1* in *P. stipitis* itself had some unexpected consequences. When grown on xylose under aerobic conditions, a *P. stipitis* strain bearing an autonomous plasmid with *XYL1* expressed under its own truncated promoter showed up to 1.8-fold higher Xor activity than the control transformant without the *XYL1* insert. Oxygen limitation led to higher Xor activity in both strains grown on xylose. When grown on glucose under aerobic or oxygen-limited conditions, the experimental strain had Xor activity up to 10 times higher than that of the control strain. Despite this increase, xylose fermentation rates did not improve significantly. This suggests that *XYL1* expression does not limit fermentation in this organism [105].

4.2 Xylitol Dehydrogenase

The second step of xylose metabolism is oxidation of xylitol to xylulose. An NAD^+-dependent enzyme, xylitol dehydrogenase (Xid), catalyzes this reaction. Xid belongs to a family of medium-chain Zn-containing dehydrogenases that are related to human sorbitol dehydrogenase and yeast Adh [107]. D-Xylose induces the genes for both Xor (*XYL1*) and Xid (*XYL2*) [108], but L-arabinose induces *XYL2* much more. Xylitol, however, does not induce either enzyme. This suggests that the two sugars share a common pathway that involves xylitol, but because xylitol does not specifically induce *XYL2*, xylose- and arabinose-specific induction pathways are implicated. Cultivation of *C. tenuis* on organic nitrogen sources such as yeast extract and peptone induced the production of Xor and Xid much more than cultivation on urea or ammonium nitrate [109]. In *P. stipitis,* the activity of Xid has been reported to decrease under anaerobic or oxygen-limited conditions [55]. In *C. shehatae* oxygen limitation has a slight positive effect on Xor and Xid activities [110]. Skoog and Hahn-Hägerdal [111] did not observe any changes in Xor or Xid activities of *P. stipitis* when cells were grown with high and low oxygen. *C. shehatae* represses XYL and XYL2 when cells are grown on glucose, but Xor and Xid activities in cells from continuous cultures grown on xylose/glucose mixtures are essentially the same as in cells grown on xylose alone [110]. However, glucose blocks some aspect of xylose metabolism because, in wild-type cells, xylose is not taken up until glucose is consumed. The roles of oxygen and cofactor levels in regulating titer and activities of Xor and Xid requires much more attention.

P. stipitis normally produces little or no xylitol but xylitol accumulates when the redox in the cell is not balanced [112]. When *PsXYL2* is introduced in *S. cerevisiae*, the resulting strain cannot ferment xylose effectively. Rather, it forms xylitol as long as a cosubstrate is present [51, 52]. *P. stipitis XYL2* has a conserved NAD^+ binding domain that contains the GXGXXG adenine-binding motif [113]. In order to avoid cofactor imbalance in the recombinant *S. cerevisiae* expressing *PsXYL1* and *PsXYL2*, Metzger and Hollenberg attempted to modify product formation by converting the NAD^+-dependent xylitol dehydrogenase into an $NADP^+$-dependent enzyme [84]. The Asp207Gly mutant or a double Asp207Gly and Asp210Gly mutant increased the K_m for NAD^+ by about nine-fold but reduced the Xid activity by 47 and 35%, respectively. Introducing a poten-

tial NADP recognition sequence (GSRPVC) from the Adh of *Thermoanaerobium brockii* into the *P. stipitis* enzyme conferred the resulting variant with equal K_m values for $NADP^+$ and NAD^+ as cofactors. This mutated enzyme can still function to support growth on xylose in the recombinant *S. cerevisiae*.

A *xyl2* mutant of *P. stipitis* CBS 6054 that would not grow on D-xylose or L-arabinose was deficient in Xid, D-arabinitol dehydrogenase (Dad), L-arabinitol dehydrogenase (Lad) and D-ribitol dehydrogenase [114]. However, it had normal L-arabinose and D-xylose reductase activities. Complementation with *PsXYL2* restored growth on D-xylose and L-arabinose. This result suggests that Xid is involved in the metabolism of both xylitol and L-arabinitol. *P. stipitis* CBS 6054 exhibits very slow growth on L-arabinose and even the wild-type strain shows only low L-arabinitol dehydrogenase (NAD^+) activity on L-arabinose, so it is possible that it does not possess a specific L-arabinitol dehydrogenase.

Hallborn et al. [115] cloned a short-chain dehydrogenase gene from *P. stipitis* CBS 6054 that has its highest activity with D-arabinitol as substrate. The D-arabinitol dehydrogenase activity is not induced by xylose but it can use xylitol as a substrate. D-Ribulose is the final product for this enzyme. This enzyme is similar to an NAD^+-dependent D-arabitol dehydrogenase cloned from *Candida albicans* [116].

4.3 Xylulokinase

The third enzyme in the xylose metabolic pathway is xylulokinase (*Xks*), which is coded for by *XKS1*. It converts xylulose to xylulose-5-phosphate. This is the first reaction in xylose assimilation that has a significant net negative free energy change; it drives assimilation under oxygen-limited conditions. This gene has been cloned from *S. cerevisiae* by complementing an *E. coli* mutant [117, 118]. There have been relatively few fundamental studies of Xks in eukaryotes. Its activity is about 13 times higher in *P. tannophilus* and 16 times higher in *C. shehatae* than in *S. cerevisiae* when all three yeasts are grown on xylose [119]. In the xylose-fermenting yeasts, Xks is induced about 10- to 20-fold by growth on xylose as compared to glucose. In *P. tannophilus* and *C. shehatae*, Xks levels are 0.9 and 1.1 IU/mg protein. Xks is not detectable during continuous cultivation of *S. cerevisiae* on glucose, but it is present during cultivation on xylulose and glucose [120]. The *P. stipitis* enzyme has a K_m value of 5.2×10^{-4} M for D-xylulose, a very high specific activity (21.4 U mg^{-1}), and requires magnesium ions for optimal activity [121].

Rodriguez-Peña et al. recently reported that an open reading frame (*YGR194c*) in *S. cerevisiae* codes for Xks [122]. The authors had previously sequenced the gene as part of the *S. cerevisiae* genome-sequencing project. Genomic comparisons showed that it has significant homology with bacterial xylulokinase genes, so they carried out a site-specific disruption to determine the physiological effects. Native *S. cerevisiae* will not grow on xylose, so purified xylulose was used as the substrate. The *xks1* mutant grew well on glucose but was incapable of growing on xylulose. When the *xks1* mutant was compliment-

ed with the *XKS1* gene, the resulting transformant grew even better than the wild type did on xylulose. However, when *XKS1* was overexpressed in a wild-type *Saccharomyces* strain, it hampered growth on xylulose as a sole carbon source. This finding requires further clarification, because it is not completely consistent with the report that expression of *XYL1*, *XYL2* and *XKS1,* in a *Saccharomyces* hybrid enhances fermentation of xylose [53].

4.4
Phosphoketolase

Various bacteria, and particularly the lactobacilli, have been reported to use phosphoketolase (EC 4.1.2.9) during the metabolism of xylose [123–126]. Phosphoketolase has been reported in a few aerobic yeasts that use xylose rapidly [127–129], and the capacity of a *Candida* sp. to accumulate lipid has been attributed to its presence [130]. However, a role for this enzyme in ethanol production from xylose has not been established.

4.5
Fermentation of Arabinose and Galactose

Some yeasts will oxidize L-arabinose for growth, and others will convert it to polyols, but few will ferment it to ethanol. Dien et al. [131] screened 116 species of yeasts for their abilities to ferment L-arabinose to ethanol. They found that *Candida auringiensis*, *Candida succiphila*, *Ambrosiozyma monospora* and *Candida* sp. (YB-2248) are capable of forming up to 4.1 g/l of ethanol from L-arabinose within 7 to 9 days when incubated with low aeration. Each of these yeasts also fermented D-xylose. Although they produced only low ethanol concentrations and rates were low, the findings are significant because they indicate that the direct conversion of L-arabinose to ethanol can occur in yeasts. These are the only yeasts known to produce ethanol from L-arabinose and they are potential candidates for further study and development.

An essential step in the metabolism of galactose is UDP-galactose-4-epimerase (EC 5.1.3.2) which converts UDP-galactose to UDP-glucose. This reaction is associated with NAD^+, which suggests that it involves sequential oxidation and reduction [132]. Skrzypek and Maleszka [133] reported cloning genomic DNA from *P. tannophilus* that is homologous to the *GAL10* gene in yeasts and *galE* gene in bacteria. Both of these genes encode UDP-galactose-4-epimerase. The *P. tannophilus* protein is most similar to the homolog from *K. lactis* protein, and it is equally induced by D-galactose or D-xylose. In *S. cerevisiae*, UDP-galactose-4-epimerase can be inactivated by D-xylose or L-arabinose [134]. Inactivation is due to the reduction of the epimerase NAD^+, which is essential for epimerase activity.

5
Respiratory Pathways in Fungi

In *P. stipitis*, fermentative and respirative metabolisms coexist to support cell growth and the conversion of sugar to ethanol [135]. The total respiration ca-

pacity of *P. stipitis* does not appear to be affected by oxygen limitation. Passoth et al. [136] observed no decrease in the respiration capacity, no increase in the respirative quotient (CO_2 production/O_2 consumption) and no change in the level of Pdh activity from *P. stipitis* cells grown on xylose or glucose after they were shifted from fully aerobic to low oxygen tensions. Moreover, respiratory activity was not repressed by fermentable sugars. This suggested that its respiration is constitutive. Subsequent studies, however, have shown that the organism has a complex respiratory system that is regulated in several ways.

The cytochrome respiratory system is the primary means for transferring reducing equivalents to oxygen in eukaryotic organisms (Fig. 6). Three complexes (I, III and IV) translocate protons across the mitochondrial inner membrane to create proton motive force. In addition, succinate dehydrogenase (complex II) transfers electrons without generating a gradient. Protons re-entering the matrix through ATP synthase generate metabolic energy. In addition to NADH dehydrogenase (complex I), higher plants and some fungi are thought to possess NAD(P)H dehydrogenases, which exist on the matrix and external sides of the inner mitochondrial membrane. Ubiquinone (UbiQ) can be reduced by NADH oxidase, succinate dehydrogenase and NAD(P)H oxidoreductase. It in turn can be oxidized by cytochrome bc_1 or by the alternative oxidase (Aox). Aox and Cox can react with molecular oxygen. Cox does so while generating a proton gradient; Aox does not. Electron transfer from Aox is blocked by salicylhydroxamic acid (SHAM). Electron transfer from cytochrome bc_1 is blocked by

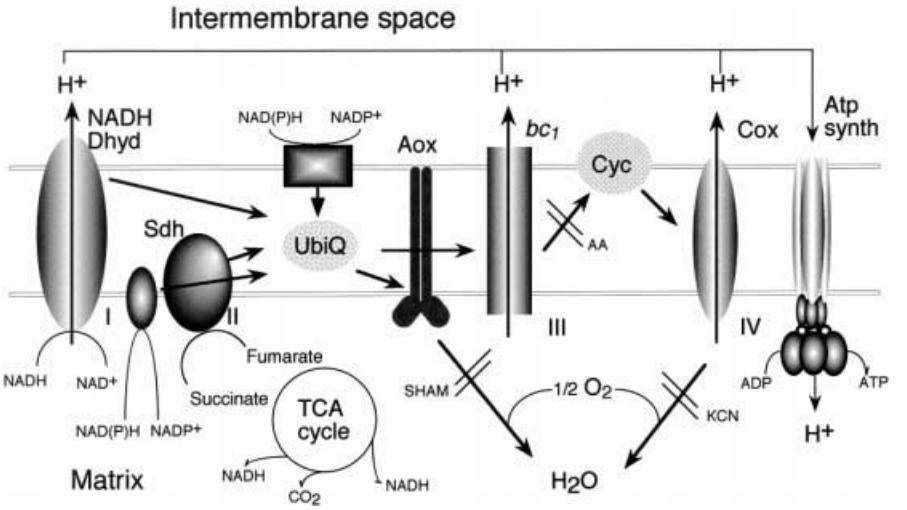

Fig. 6. Electron transport in plants and fungi. Three complexes (*I*, *II* and *IV*) translocate protons to gradient across the mitochondrial membrane: *Complex I* NADH dehydrogenase; *complex II* succinate dehydrogenase; *complex III* cytochrom bc_1; *complex IV* cytochrome *c* oxidase (Cox). *Cyc* Cytochrome *c*; *UbiQ* ubiquinone; *Aox* alternative oxidase; *SHAM* salicylhydroxamic acid; *AA* antimycin A; *KCN* potassium cyanide. (From Vanlerberghe and McIntosh [137])

antimycin A (AA). Electron transfer from Cox is blocked by cyanide (KCN). Electrons are accepted by cytochrome *c* via cytochrome complex bc_1 (III) and donated to cytochrome *c* oxidase (IV).

Cox is the primary oxidase in eukaryotes. However, Aox is present in certain yeasts, fungi and plants. It can act as an ultimate electron acceptor. Aox links only to complex I. When Aox is active, two of the three possible sites for coupling electron transfer to proton translocation and energy generation are bypassed as compared to the bc_1 cytochrome pathway. However, some energy can still be generated as a consequence of proton translocation at complex I. The Aox can reduce molecular oxygen in a four-electron transfer step and Aox is activated when electron transfer through the cytochrome pathway is constricted [137].

In 1995 Jeppsson et al. [138] reported that *P. stipitis* has an Aox that resists cyanide but is sensitive to SHAM. The presence of an Aox distinguishes *P. stipitis* not only from *S. cerevisiae*, but also from the pentose utilizing yeasts *Candida utilis* and *P. tannophilus.* Several other yeasts, fungi and higher plants possess Aox systems [139–142]. In the yeast *Hansenula anomala*, Aox is induced in response to the respiratory inhibitor AA [143], suggesting that it is formed in response to increasing intracellular reductant. In plants, intracellular pyruvate and the redox level regulate activity of the alternative respiratory system [144]. Jeppsson et al. [138] suggested that the Aox of *P. stipitis* is responsible for the very low level of xylitol production observed during fermentations of xylose by this yeast. It may scavenge for reductant, provide an alternative pathway for terminal oxidation, and be responsible for the oxidation of NAD(P)H. It is possible that as long as O_2 is available, the Aox provides a route for the utilization of reductant in *P. stipitis.*

Indirect evidence suggests that the Aox of *Hansenula anomala (= Pichia anomala)* has a similar function to the SHAM-sensitive Aox in *P. stipitis*. First, respiratory inhibitors induce the *H. anomala* Aox just as they do in *P. stipitis* [145]. In fact, the *H. anomala* gene was cloned as a cDNA that was induced in response to inhibition by antimycin A [146]. The *H. anomala* Aox is closely related to a family of enzymes that code for Aox in plants. One of the most closely related is the Aox of *Sauromatum guttatum* (voodoo lily) [147]. Heterologous expression of the *S. guttatum* protein in the mitochondrion of the fission yeast, *S. pombe*, imparts in it the capacity for cyanide-insensitive respiration [148]. Monoclonal antibodies made to the *S. guttatum* Aox cross-react with the *H. anomala* protein and with the cyanide-resistant, SHAM-sensitive Aox of *Neurospora crassa* [149]. Moreover, as in the case of *P. stipitis*, impairing the *N. crassa* cytochrome system induces its Aox [150]. One can observe close sequence similarity between the *S. guttatum* protein and similar Aox proteins found in the mitochondria of other plants such as *Arabidopsis* [151].

The branch point between the Aox and the cytochrome respiratory chain arises at the ubiquinone pool. These terminal oxidases can be identified by using respiratory inhibitors and are divided as different groups according to the sensitivities or resistances to the inhibitors. Alternative respiration is activated by stress stimuli factors that constrict the electron flow through the cytochrome pathway [137]. However, the roles and the compositions of these pathways are

not fully understood in yeasts. In *S. guttatum*, a high respiration rate through a SHAM-sensitive pathway quickly releases energy as heat to volatilize special compounds to attract pollination insects [152]. Thus, the alternative respiration has been considered as a control system to balance the respiratory carbon metabolism, demand for the reducing equivalents and energy.

P. stipitis may use different electron-transport chains to recycle reducing equivalents in supporting cell growth on xylose and glucose. When CBS 6054 is grown on xylose in the presence of the cytochrome pathway inhibitor, cyanide, 0.45 g/g ethanol is produced [138]. However, when the alternative respiratory pathway is blocked with SHAM on xylose, *P. stipitis* produces only 0.35 g/g of ethanol. In addition, the presence of SHAM does not inhibit the respiration on xylose-grown cells but it does show 11% of inhibition on glucose-grown cells. Therefore, separate roles for each of the electron-transport pathways are implicated in supporting cell growth on xylose. Previous evidence demonstrated that cells of wild-type *P. stipitis* CBS 6054 grown on xylose have a 1.7-fold higher oxygen uptake rate than the cells grown on glucose. However, the CBS 6054 cells grown on xylose has 1.5-fold lower cytochrome *c* oxidase activity. It seems likely that metabolism of the two sugars is supported by different respiratory pathways. If xylose metabolism depended in some way on the activity of the Aox, this might explain its linkage to respiration.

A mutant strain of *P. stipitis,* FPL-061, was obtained by selecting for growth on L-xylose in the presence of respiratory inhibitors [153]. This strain displays lower cell yield and relatively higher specific ethanol production rate than its parent, CBS 6054. Because it shows a lower cell yield, it is probably deficient in some aspect of energy generation. With a mixture of glucose and xylose, the FPL-061 mutant produced 30 g ethanol/l with a yield of 0.42 g ethanol/g sugar consumed. By comparison, CBS 6054 produced 26 g ethanol/l with a yield of 0.35 g/g under the same conditions [154]. Fermentation by the mutant was most efficient at an aeration rate of 9.2 mmoles O_2 L^{-1} h^{-1}. This is significantly higher than the optimum aeration rate reported for wild-type *P. stipitis*. The *P. stipitis* xylose fermentation is best under low aeration conditions [155]. This is probably attributable to the obligate hypoxic induction of essential fermentative enzymes coupled to the inability of the yeast to grow and synthesize new proteins under anaerobiosis. The optimal aeration rate for ethanol production by *P. stipitis* has been reported as 1.5 [156] to 1.75 [157] mmol L^{-1} h^{-1}. At high aeration rates (22 mmol O_2 L^{-1} h^{-1}) the FPL-061 mutant cell yield was less than that of the parent. At low aeration rates (1.1 to 2.5 O_2 L^{-1} h^{-1}), cell yields were similar, the ethanol formation rates were low, and xylitol accumulation was observed with both strains. Both strains respired the ethanol once sugar was exhausted. It can be inferred from the results that the mutant, *P. stipitis* FPL-061, is deficient in its oxygen uptake system. Its optimum aeration rate for ethanol production is higher than the wild type, and its cell yields are lower at higher aeration rates.

Harrod et al. selected for respiratory deficient strains of *P. tannophilus* [158]. In *P. tannophilus,* utilization of malic and succinic acid is repressed by glucose. The authors characterized strains with mutations in two hexokinases and a glucokinase for their growth on glucose in the presence of L-malic and succinic

acid. They found increased specific utilization of malic and succininc acid in mutants deficient in hexokinase A. This type of mutation is similar to that observed with carbon catabolite derepressed strains of *Saccharomyces.*

6 Regulatory Mechanisms

The mechanisms for regulating xylose and glucose fermentation differ among yeasts. In *S. cerevisiae,* glucose represses respiration and stimulates fermentation. Aeration is essentially irrelevant to *S. cerevisiae* when it is provided with excess glucose [159]. *S. cerevisiae* induces enzymes for fermentative metabolism and increases its glycolytic flux in the presence of fermentable sugars [160]. Glucose induces transcription of *PFK*, pyruvate kinase (*PYK*) and pyruvate decarboxylase (*PDC*) [62, 161]. Full induction of *PDC* appears to be attributable to accumulation of hexose and triose phosphate [162]. Proper transcriptional regulation of these genes depends on the formation of glucose-6-phosphate by any one of the three hexose kinases. *S. cerevisiae* becomes fermentative at high flux rates in the presence of oxygen even when glucose is not present in excess. This is a phenomenon known as the Crabtree effect [163]. Yeasts in which glucose represses respiration and induces fermentation even when oxygen is not limiting are Crabtree-positive. Yeasts in which glucose does not repress respiration and induce fermentation are termed Crabtree-negative [61].

Sierkstra et al. [164] studied the induction of glycolytic enzymes in glucose and galactose limited continuous cultures of *S. cerevisiae* to understand better the Crabtree effect. They increased the dilution rate (D) until wash out occurred, and they simultaneously kept the dissolved oxygen tension at 20% so that cultures did not become oxygen limited. With *S. cerevisiae* cells grown on 1% glucose, enzyme levels for Adh1, Adh2, Pfk and Pg1 decreased steadily with increasing dilution rate, and transcript levels decreased in parallel with activity levels. Acetaldehyde and acetate increased with ethanol production and the respiratory quotient. Similar results were obtained with galactose cultures.

These results suggest that in *S. cerevisiae,* glucose-limited cultures with excess oxygen produce ethanol as a consequence of overflow metabolism at the pyruvate branch point rather than in response to the induction of glycolytic enzymes. Because the onset of ethanol production occurs at the same dilution rate with glucose and galactose, the Crabtree effect is not thought to be glucose specific. The overflow model suggests that the accumulation of acetate inactivates electron transport and forces the diversion of pyruvate into fermentation. These results indicate that flux partitioning at the level of pyruvate is regulated mainly by allosteric factors [165].

The essential steps depicting the branch point between fermentation and respiration are summarized in Fig. 7. Pyruvate dehydrogenase (Pdh) and Pdc compete for pyruvate. Either pyruvate can be oxidized via Pdh to form acetyl CoA, or it can be decarboxylated by Pdc to form acetaldehyde. The partitioning of pyruvate at this step is kinetically controlled. In *S. cerevisiae*, isolated mitochondria and purified Pdh exhibit much higher affinity (lower K_m) for pyruvate

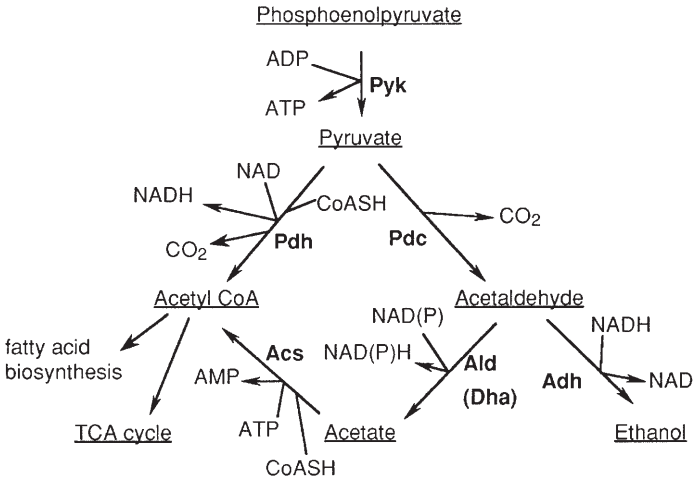

Fig. 7. Branch point between fermentation and respiration. At low pyruvate flux, the low K_m of the Pdh complex for pyruvate results in oxidative decarboxylation to form acetyl CoA and NADH. The acetyl CoA can then can go into energy generation (via respiration) or fatty acid synthesis. At high glycolytic flux, pyruvate accumulates, and the higher V_{max} of Pdc favors acetaldehyde formation and ethanol production. Accumulation of acetate can interfere with mitochondrial function. *Pyk* Pyruvate kinase; *Pdh* pyruvate dehydrogenase; *Pdc* pyruvate decarboxylase; *Ald* (Dha) aldehyde dehydrogenase; *Adh* alcohol dehydrogenase; *Acs* acetyl CoA synthetase. (Taken from Postma et al. [169])

than does Pdc. At low glycolytic flux, pyruvate concentrations are low and the lower K_m of Pdh for pyruvate favors formation of acetyl CoA. As the flux exceeds the maximum capacity of Pdh, pyruvate begins to accumulate, and metabolites are shunted into acetaldehyde formation. Allosteric regulation of Pdc also plays a role. Pdc activity exhibits cooperativity with respect to pyruvate. At higher pyruvate levels, the activity of Pdc increases. With higher glycolytic flux and higher pyruvate levels, Pdc formation of acetaldehyde is favored because of the higher V_{max} of this reaction. Ethanol production begins, but as long as respiration is active, a portion of the acetaldehyde is oxidized to acetate via acetaldehyde dehydrogenase (Ald). The partitioning between acetate and ethanol depends ultimately on the relative abundance of reductant and oxygen. Acetate can be recovered for respiration through acetyl CoA synthetase (Acs), but under the conditions favoring acetaldehyde formation in comparison to Pdh, this route requires an extra ATP. Acetate can accumulate intracellularly if Acs activity is not sufficient.

When the *ScPDC1* gene is over expressed in *S. cerevisiae*, the overproduction of Pdc affects flux distribution at the pyruvate branch point by influencing competition for pyruvate between Pdc and the mitochondrial Pdh complex [166]. In respiratory cultures *of S. cerevisiae*, Pdc overproduction does not effect the maximum glycolytic capacity. At high intracellular concentrations, pyruvate is predominantly metabolized via Pdc, but at low pyruvate concentration, it is metabolized through Pdh [161]. The purified *S. cerevisiae* Pdh has a much lower

Km for pyruvate than does Pdc [167]. Overproduction of Pdc results in a shift to fermentation at a lower dilution rate than observed with wild-type cells [166]. However acetate is essential for *pdc* cells growing on glucose in defined medium [168]. The presence of pyruvate and acetate in the cell uncouples ATP production, leading to a reduction in cell yield [169]. These observations are consistent with organic acid inactivation of respiration.

P. stipitis and *C. shehatae* are Crabtree-negative yeasts. Glucose (or xylose) does not induce fermentation in these organisms. Rather, fermentation is "activated" in response to anaerobiosis or oxygen limitation. The induction of fermentative enzymes results from a reduction in the oxygen tension rather than from an increase in the glycolytic flux, as in the case of *S. cerevisiae*.

In *P. stipitis* and *C. shehatae*, fermentation is regulated by the oxygen supply or by respiratory activity. Alexander et al. [110] showed that in *C. shehatae*, as the cells become oxygen limited and fermentative activity increases, Adh activities increase 10-fold. A similar effect has been observed by du Preez et al. [55] *P. stipitis* requires well-controlled low oxygenation (1.5 – 1.75 mmol/h) to grow and ferment xylose effectively in batch culture [135]. When it grows in oxygen-limited continuous culture, the specific ethanol production rate reaches a maximum at an O_2 transfer rate below 1 mmol/l h. When Skoog and Hahn-Hägerdal [111] shifted *P. stipitis* from aerobic to oxygen-limited conditions, Pdc activity increased three- to five-fold. As oxygen became limiting, the malate dehydrogenase (Mdh) level decreased.

Passoth et al. [136] observed rapid 10-fold increases in Pdc and Ald activities, and a 20-fold increase in Adh activity, following a shift from fully aerobic to oxygen-limited conditions in *P. stipitis* grown on either glucose or xylose. Apparently glucose is required for full expression of fermentative genes in *C. shehatae* because Adh is induced roughly two- to threefold when it is grown on glucose as opposed to xylose [110]. These results indicate that the fermentative pathway for pyruvate metabolism is induced (or derepressed) by oxygen limitation in *P. stipitis* and *C. shehatae*. In the case of the latter organism, some increase in fermentative processes might be induced by glucose.

7 Pyruvate Partitioning

C^{13} and ^{31}P NMR studies show that *S. cerevisiae* suspensions metabolizing glucose have close to twice the level of nucleoside tri- and diphosphates (mainly ATP and ADP) found in *P. stipitis* suspensions metabolizing either glucose or xylose [170]. This observation suggests that either sugar metabolism in *S. cerevisiae* occurs at a higher rate or it generates more energy. Probably it is attributable to a higher glycolytic rate because the overall cell yield with wild-type *P. stipitis* is the same or even higher than that observed with *S. cerevisiae*. This implies that the reactions leading up to pyruvate function at a lower rate in *P. stipitis* than in *S. cerevisiae*. Accordingly, the respiratory and fermentative metabolic activities must be attuned to these levels. With *S. cerevisiae*, the enzymes for transport and glycolysis are induced in response to glucose. This results in a high glycolytic flux. With pentose-fermenting yeasts, the flux with glucose or xylose is never as great.

7.1
Pyruvate Decarboxylase

Pyruvate decarboxylase (Pdc) converts pyruvate to acetaldehyde, which is then reduced to ethanol by Adh. This enzyme is found in microorganisms whose predominant fermentation product is ethanol. Pyruvate is the step at which partitioning between acetyl CoA (leading to respiration) and acetaldehyde (leading to fermentation) occurs through the activities of Pdh and Pdc, respectively [171]. Although the pathways and regulation now appear to be much more complex than the kinetic regulation originally envisioned, regulation of Pdc activity seems to be instrumental in determining metabolite flow. In *S. cerevisiae*, Pdc activity is induced in response to a pulse of glucose, whereas activities of Adh, acetaldehyde dehydrogenase (Ald) and acetyl CoA synthetase remain unchanged [168].

Three *PDC* structural genes have been characterized in *S. cerevisiae*: *ScPDC1* [172], *ScPDC5* [173, 174], and *ScPDC6* [175, 176]. The three structural genes are expressed differently at the transcriptional level [177]. In *P. stipitis*, Pdc activity is induced ten-fold as oxygen becomes limited [136]. In contrast to *S. cerevisiae*, the Pdc activity of *P. stipitis* is not inhibited by phosphate and does not appear to be induced by a signal from glycolysis.

Two highly divergent *PDC* genes are present in *P. stipitis* CBS 6054 [178]. *PsPDC1* codes for a protein of 597 amino acids, which is 37 residues longer than that found in the isozymes of *S. cerevisiae*. There is 69.4% identity in the amino acid sequence and 63.3% identity at the DNA level. *PsPDC2* has 67.9% identity to *ScPDC1* at the amino acid level and 62.2% at the nucleotide level. The 5′ regions of both *P. stipitis* genes include two putative TATA elements that make them similar to the PDC genes from *S. cerevisiae*, *Kluyveromyces marxianus* and *Hanseniaspora uvarum*. An 81 nucleotide (27 amino acid) loop is present in the middle of *PsPDC1* which is not present in other yeast Pdc genes. Amplification of this region from genomic DNA and mRNA has shown that it is not an intron. Preliminary data demonstrate that the two *PDC* genes in *P. stipitis* are regulated by various carbon sources and by oxygen availability [179]. Transcripts of *PsPDC1* are detected when grown on glucose, glycerol and xylose, but the level is highest on glucose. *PsPDC2* appears to be expressed only on glucose but not on xylose or glycerol. The expression levels of both genes increases significantly when the cells are shifted from aerobic to anaerobic conditions.

7.2
Alcohol Dehydrogenase

S. cerevisiae has four isozymes for alcohol dehydrogenase (Adh). Adh1 is responsible for reduction of acetaldehyde to ethanol [180–182]. Adh2 is highly repressed by glucose [183]. Its function is to oxidize ethanol to acetaldehyde [184]. *ADH3* codes for a mitochondrion-targeted enzyme presumably involved in ethanol oxidation [185], and *ADH4* displays no significant similarity with any other characterized yeast *ADH* gene [186].

P. stipitis CBS 6054 has at least two genes that code for Adh [187]. The predicted amino acid sequences of *PsADH1* and *PsADH2* are 80.5% identical to one another and are 71.9 and 74.7% identical to *S. cerevisiae ADH1*. They also show high identity with other group I Adh proteins. The PsAdh isozymes are presumably localized in the cytoplasm as they do not possess the amino terminal extension of mitochondrion-targeted ADHs. *PsADH1* has both fermentation and respiration functions. *PsADH1* is the principal gene responsible for ethanol production because its disruption results in slower growth, lower ethanol production, and much greater xylitol production under oxygen-limited conditions; whereas the disruption of *PsADH2* has none of these effects. Disruption of *PsADH1* in *P. stipitis* causes this yeast to make even more xylitol than does *C. shehatae* or *P. tannophilus*. This could result from reductant imbalances. Cho and Jeffries [187] hypothesized that in *P. stipitis*, xylitol production is normally low because PsAdh1 suppresses the intracellular level of NADH. When *PsADH1* is disrupted, NADH accumulates. Accumulation of NADH would shift the equilibrium of the XDH-mediated reaction to favor xylitol over xylulose formation. PsAdh1 and PsAdh2 isozymes appear to be equivalent in their ability to convert ethanol to acetaldehyde and either is sufficient to allow cell growth on ethanol. The *PsADH* double disruptant cannot grow on ethanol, but it still produces ethanol from xylose at about 13% of the rate seen in the parental strain when it is grown on xylose under oxygen-limited conditions. This implies separate paths for ethanol fermentation and respiration.

PsADH2 is not expressed under aerobic or fermentative conditions unless *PsADH1* is disrupted. The inability of the *Psadh* double disruptant to grow on ethanol under full aerobiosis suggests that no other Adh activities are present under these conditions, but the continued ethanol production under oxygen-limited conditions implies that other isozymes exist. Indeed, evidence of a third Adh can be seen in some Southern blots of *P. stipitis* DNA. The gene for a third isoenzyme may be present but not expressed on ethanol under aerobic conditions.

Regulation of the two *PsADH* genes in *P. stipitis* reveals a very different mechanism from what is reported in *S. cerevisiae* [188]. *PsADH1* transcription increases tenfold when cells are shifted from fully aerobic to oxygen-limited conditions. This correlates well with observed changes in enzyme activity and supports independent observtions by Passoth et al. [136] However, transcripts of *PsADH2* are not detectable under either aeration condition. β-Galactosidase activity from a *PsADH1::lacZ* fusion construct showed that expression of *PsADH1* increased as aeration decreased. These results suggest that *PsADH1* expression is affected by oxygen. Oxygen regulation may be mediated by heme. The level of *PsADH1* transcript in cells grown on xylose under oxygen-limited conditions in the presence of heme was about one-tenth the level of its transcript in cells grown in its absence [188]. The disruption of *PsADH1* caused a dramatic increase in *PsADH2* expression on non-fermentable carbon source under fully aerobic conditions, providing a provisional indication that the expression of *PsADH2* is subject to feedback regulation under these conditions.

7.3
Pyruvate Dehydrogenase

The pyruvate dehydrogenase (Pdh) complex catalyzes the conversion of pyruvate to acetyl CoA for respiration. In both prokaryotes and eukaryotes, this multisubunit complex is composed of multiple copies of three enzymes and as many as five proteins. In the central core are 60 subunits of E2 (acetyl-CoA dihydrolipoamide *S*-acetyl transferase). Around this core are multiple copies of $E_{1\alpha}$ and $E_{1\beta}$ (pyruvate dehydrogenase), E3 (dihydrolipoamide dehydrogenase) and protein X (unknown function) [189–192]. Deletion of the $E_{1\alpha}$ gene in *S. cerevisiae* results in complete loss of Pdh activity [193]. Preliminary studies from our laboratory indicate that the *P. stipitis PDH* $E_{1\alpha}$ homolog, *PsPDH* [194], like the *PDH* of *S. cerevisiae*, is not transcriptionally regulated following shifts in carbon source or aeration. This correlates with the observation by Passoth et al. [136] that activity levels do not change following shifts in aeration. In practice, it is difficult to manipulate the kinetic parameters of Pdh due to the complexity of this multienzyme complex. Our research has therefore focused on altering the activities of Pdc and Adh.

8
Glucose and Oxygen Regulation

The discussion in this section focuses on the major regulatory elements found in *S. cerevisiae*. Genetic studies with other yeasts and various higher eukaryotes have shown that homologous genes with similar functions exist in other organisms, so the basic mechanisms should be similar for *S. cerevisiae* pentose-fermenting yeasts. Most research has been directed towards identifying and dissecting the regulatory mechanisms in *S. cerevisiae*. Relatively little effort has been made to put them to use in improving fermentation properties. Trumbly [195] and more recently Gancedo [196] have reviewed glucose repression. The key elements include the glucose transporter analogs Snf3 and Rgt2, which appear to act as glucose sensors, the protein kinase Snf1, whose activity is required for derepression of many glucose-repressible genes, and the Mig1 repressor protein, which binds to the upstream regions of glucose-repressible genes. Repression by Mig1 requires the activity of the Cyc8 and Tup1 general repressor proteins.

Oxygen regulation is mediated through heme biosynthesis. In *S. cerevisiae*, glucose and oxygen regulation are intertwined because Mig1 represses activities of Hap1 and Hap2/3/4/5, the transcriptional activators for genes of respiratory metabolism. In the absence of heme, Rox1 represses genes related to heme, sterols and unsaturated fatty acids [197, 198].

8.1
Glucose Repression

Glucose and fructose are the preferred carbon sources for *S. cerevisiae* and many other yeasts. When these sugars are present in the medium, enzymes for

using other sugars are repressed. Although glucose can effect expression at several levels, the main effect takes place at the transcription [196].

8.1.1 *Adr1*

The transcription factor Adr1 is required to fully activate expression of *ADH2* when glucose becomes depleted (Fig. 8). Adr1 is a zinc finger protein belonging to the Cys2-His2 family. It binds a 22 bp palindromic sequence in the *ADH2* promoter [196]. The activity of Adr1 is itself modulated. cAMP-dependent protein kinase (cApk) has been implicated in its inactivation [199]. The protein kinases Snf1 (Ccr1) and Sch9 increase *ADH2* expression independently of both Adr1 and cApk [200]. Adr1 may be a more general regulator [201]. An Adr1 homolog appears to regulate methanol oxidase (*MOX*) expression in *Hansenula polymorpha* [202], and a similar regulatory system may also exist in *P. stipitis*. Like *S. cerevisiae*, it has been shown to possess at least two genes for Adh. *PsADH1*, which is primarily responsible for ethanol production, is expressed on glucose and xylose and induced under oxygen-limited conditions. The other (*PsADH2*) is expressed only under fully aerobic conditions or in a *Psadh1* background [188]. The 5′-UTR region of *PsADH1* has three putative binding sites for Adr1 in the positive strand at positions –234, –244 and –378. The 5′-UTR region of *PsADH2* has six potential binding sites for Adr1 in the positive strand within

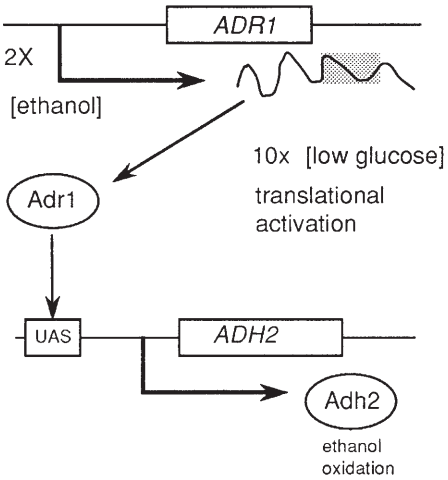

Fig. 8. Multiple modes of Adh2 regulation. Production of the ethanol oxidizing enzyme, Adh2, is regulated through multiple modes. In the presence of ethanol, mRNA levels of the transcriptional activator, Adr1, increase approximately twofold. Translational activation also occurs because when glucose concentrations are low production of the Adr1 protein increases about tenfold even though the mRNA levels do not change significantly. The glucose translational activation sequences are apparently embedded within the coding region of the *ADR1* mRNA

450 bp of the coding sequence and the negative strand contains numerous potential Adr1 binding sites [187]. It remains to be determined whether these putative sites are involved in glucose regulation or some other response.

8.1.2 *Snf1*

The *SNF1* (*CAT1*) gene of *S. cerevisiae* encodes a protein-serine/threonine kinase that is required for derepression of gene expression in response to glucose limitation [203]. It is associated with several other regulatory proteins [204]. Snf1 is shared by various circuits and is a central element in the regulatory process. Snf1/Cat1 is not active in the presence of glucose [196]. One of the main roles of Snf1 is to relieve repression by the Mig1 complex, but it is also required for the operation of transcription factors such as Adr1. A homolog of the *SNF1* gene (*KlSNF1*) is also found in *K. lactis*. In this yeast, galactose utilization is repressed by glucose and derepressed by its absence. Full induction requires *KlSNF1*. Full repression requires a homolog of the *S. cerevisiae MIG1* gene product, *KlMIG1* [205]. The interested reader should consult Gancedo for a more complete discussion of the Snf1 regulatory system in yeast [196].

8.1.3 *Mig1*

The *S. cerevisiae* gene *MIG1* and the *Aspergillus nidulans* gene *CREA* encode Cys2-His2 zinc finger proteins that mediate glucose repression in several genes by binding to their promoters and recruiting the general repression complex Ssn6-Tup1 [206–209]. *MIG1* binding requires an AT rich region 5' to a GC box with the consensus sequence (G/C)(C/T)GGGG (Fig. 9). Mig1-like proteins are found widely in eukaryotic cells where they form a family of related DNA binding proteins with highly conserved regions for binding GC boxes. Genes similar to *MIG1* are found in the yeasts *K. lactis*, *Kluyveromyces marxianus, S. pombe* and *C. albicans* [210], and in the ascomyceteous fungus, *Aspergillus nidulans* [211]. Mig1 appears to have a similar function among the various organisms.

In *S. cerevisiae*, Mig1 is involved in glucose repression of galactose [197] and sucrose [212] utilization and respiration. Mig1 is active only in the presence of glucose, and it is inhibited when phosphorylated by the Snf1 (Cat1) protein kinase [213]. The *MIG1* and *CREA* gene products have many similar roles in mediating glucose repression among fungi. The *CREA* gene product appears to be involved in the carbon catabolite repression of cellobiohydrolase I from *Trichoderma koningii* [214], and in the carbon catabolite repression of xylanase production by *A. nidulans* [215]. The *CREA* gene product is well conserved between *A. nidulans* and *A. niger* [216]. In *K. lactis*, glucose represses the galactose utilization through a Mig1/Snf1 pathway [205], and a corresponding gene product may be involved in *P. stipitis* regulation as well.

The subcellular localization of Mig1 is also regulated by glucose. Mig1 is imported into the nucleus following the addition of glucose and is rapidly transported back to the cytoplasm when glucose is removed. Because Mig1 represses

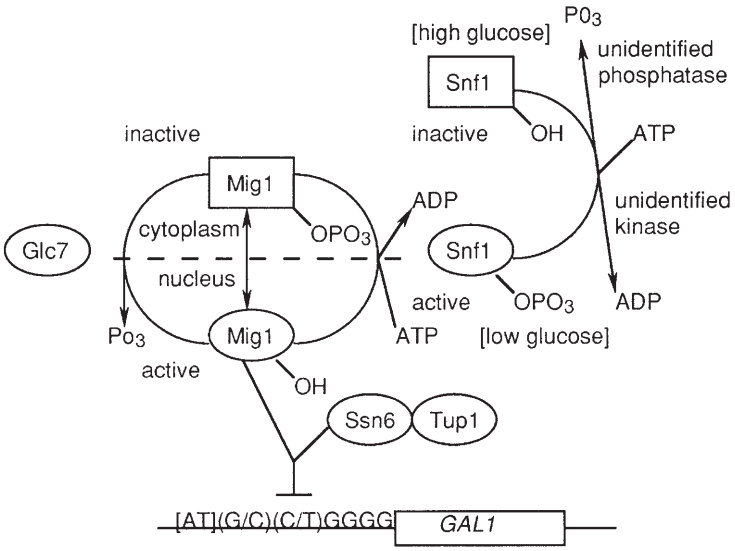

Fig. 9. Activation of the transcriptional repressor, Mig1. Mig1 represses the transcription of genes involved in the metabolism of carbohydrates other than glucose. Relief of Mig1 repression requires the activity of the serine-threonine protein kinase, Snf1. Within minutes after the removal of glucose, Mig1 is phosphorylated and transported from the nucleus to the cytosol where it can no longer interact with its DNA consensus sequence. Snf1 activity is probably regulated by phosphorylation, but the kinases and phosphatases that act on it are as yet unidentified. Mig1 functions by recruiting the Ssn6–Tup1 general repressor complex. Glc7 is a protein phosphatase that has been implicated in the activation of the Mig1 repressor complex [196, 213, 217]

transcription by binding to DNA, its nuclear location in the presence of glucose is consistent with that role. An internal region of the protein enables glucose-regulated nuclear import and export. Changes in the phosphorylation status of Mig1 coincide with changes in its localization, suggesting a possible regulatory role for phosphorylation. These results suggest that a glucose-regulated nuclear import mechanism controls the activity of Mig1 [217].

Because of its direct DNA-binding role in mediating glucose repression, researchers have examined Mig1 as a target for engineering glucose derepression. Disruption of Mig1 alleviated glucose repression of maltose (*MAL*) and sucrose (*SUC*) genes in a haploid strain of *S. cerevisiae* [218], but glucose regulation was more stringent in a polyploid strain. Deletion of Mig1 alleviated repression of invertase (*SUC1*), but it did not affect maltose genes because they require induction. In polyploid strains it is necessary to carry out multiple disruptions in order to eliminate activity. In a follow-up study, the same research group therefore examined the feasibility of using an antisense construct to silence *MIG1*, but this was not as effective as targeted disruption [219].

8.1.4
Ssn6 and Tup1

Repression by Mig1 requires the activity of the Ssn6 and Tup1 proteins. Ssn6 and Tup1 form a general transcriptional repressor complex that regulates expression of aerobically repressed hyopxic genes and glucose repressed genes in addition to mating type genes and genes controlling cell flocculence [220]. Mutations in either *TUP1* or *SSN6* (= *CYC8*) eliminate or reduce glucose repression of many repressible genes and induce other phenotypes. *SSN6* codes for a 107 kDa protein, which lacks consensus sequences for DNA-binding domains [221]. The *TUP1* gene codes for a 78 kDa protein containing six repeats of about 43 amino acids each [222]. The absence of DNA-binding motifs and the presence of repeated domains suggest that the Ssn6 and Tup1 proteins function via protein–protein interaction with transcriptional regulatory proteins [223]. Keleher et al. [224] have proposed that the Ssn6–Tup1 complex is a general repressor of transcription recruited to target promoters by various sequence-specific DNA-binding proteins in yeast. Because of their multiple roles in regulation, deletion of Ssn6 and Tup1 can have deleterious pleotropic effects.

8.2
Oxygen Regulation

The essential elements of oxygen regulation of *S. cerevisiae* genes are shown in Fig. 10. This figure is based on Zitomer and Lowry [225], Kwast et al. [63] and others as noted in the text. Heme is central to oxygen sensing in all organisms from bacteria to humans [226]. Oxygen regulation in *S. cerevisiae* is linked to glucose repression, because a central activator, the *h*eme *a*ctivated *p*rotein, Hap1 (= Cyp1) and the Hap 2/3/4/5 complex are repressed in the presence of glucose by Mig1 and the general repressor complex Ssn6–Tup1. Rox1 (*r*egulation by *Ox*ygen) represses the transcription of genes that are required under hypoxic conditions.

8.2.1
Hap1

Several features suggest that Hap1 is a general sensor of the redox state of the cell and that it is required for the full-level expression of aerobic genes [227]. Hap1 activates the transcription of oxygen-dependent, heme-dependent genes in *S. cerevisiae* [228, 229]. It promotes transcription of the structural genes for cytochromes, oxidative phosphorylation and heme, sterol and unsaturated fatty acid biosynthesis. Genes positively affected by Hap1 include *CYC1*, *CYC7*, *CYB2*, *ERG11* [230, 231], *COR2* [232] and *HEM13* [233, 234].

A single DNA-binding domain within Hap1 specifies binding to the two target sites of different sequence, UAS1 of *CYC1* (iso-1-cytochrome c) and *CYC7* (iso-2-cytochrome c). Hap1 contains a cysteine-rich zinc finger (residues 1–148), which is responsible for DNA binding, and an acidic domain (residues 1307–1483), which is essential for activation of transcription [235]. Internal

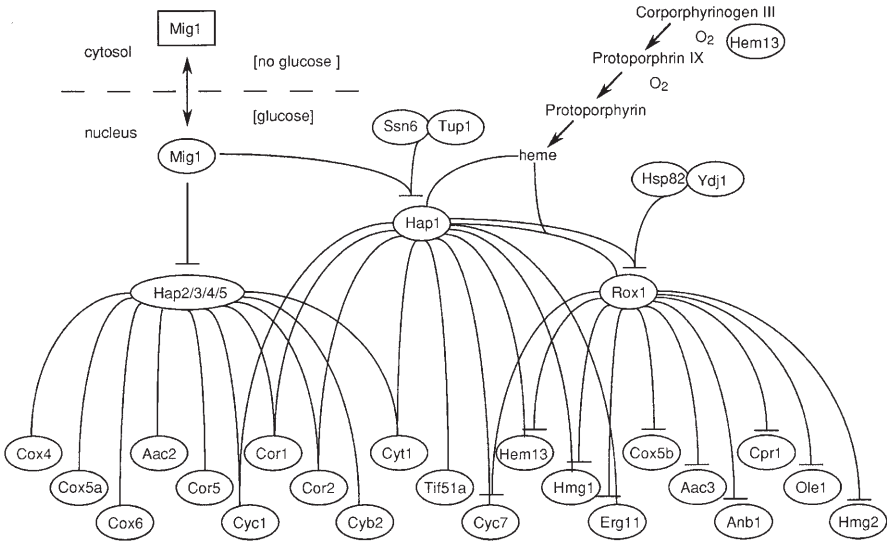

Fig. 10. Signal transduction in regulation of yeast respiration. Glucose and oxygen affect the biosynthesis of respiration-related proteins through the actions of Hap1 and heme, respectively. In the absence of glucose, Mig1 is phosphorylated by Snf1 and translocated into the cytoplasm so that it no longer represses Hap1 and Hap2/3/4/5 activities. Oxygen is required for two steps of heme biosynthesis, and the presence of heme is essential for the activation of transcription by Hap1. The heme–Hap1 complex activates transcription of Rox1, but heme has a repressive effect when interacting with Rox1. This interaction forms a feedback loop that regulates transcription of Hem13 and heme production. The major function of Rox1 is to repress genes related to the biosynthesis of heme, sterols and unsaturated fatty acids. Several proteins such as Cox5a/Cox5b, Cyc1/Cyc7 and Aac2/Aac3 and the translationfactors eIF-5a/eIF-5b have paired isoforms that are differentially expressed [63, 196, 225, 240]. *Cox4* Cytochrome c oxidase subunit IV; *Cox5a* cytochrome c oxidase subunit Va; *Cox6* cytochrome c oxidase subunit V; *Aac2* mitochondrial adenine translocase; *Cor5* ubiquinol cytochrome *c* reductase subunit XIII; *Cyc1* iso-1-cytochrome *c*; *Cor1* ubiquinol cytochrome *c* reductase subunit II; *Cyb2* cytochrome *b*2; *Cyt1* cytochrome *c*1; *Tif51a* eIF-5a; Cyc7 iso-2-cytochrome *c*; *Hem13* coproporphyrinogen III oxidase; *Hmg1* 3-hydroxy-3-methylglutaryl CoA reductase; *Erg11* cytochrome P_{450} lanosterol 14α-demethylase; *Cox5b* cytochrome *c* oxidase subunit Vb; *Aac3* mitochondrial adenine translocase; *Anb1* eIF-5b; *Cpr1* NADPH cytochrome P_{450} reductase; *Ole1* Δ-9 fatty acid desaturase; *Hmg2* 3-hydroxy-3-methylglutaryl CoA reductase

deletions in Hap1 have opposite effects because of its binding at two dissimilar sites in UAS1 of *CYC1* and *CYC7* [236]. Turcotte and Guarente [237] proposed a model involving different cofactor proteins that might exist for each site. Heme promotes Hap1 dimerization in yeast cells, thereby leading to transcriptional activation, and the regulatory domain acts by preventing dimerization of Hap1 in the absence of heme (Fig. 11) [238].

Verdiere et al. [233] suggested that Hap1 is an efficient activator in heme-depleted cells, that it exerts both positive and negative regulatory effects, and that the nature of the regulatory function of Hap1 depends on the target gene. For a given gene, the presence or absence of heme or oxygen reverses the sense of

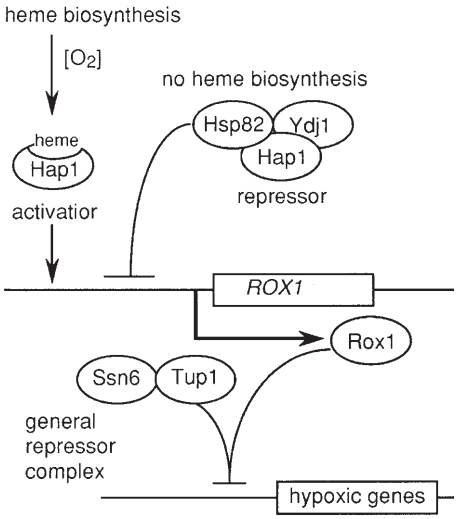

Fig. 11. Hap1 can form a transcriptional activator or repressor complex. In the presence of heme, Hap1 is a transcriptional activator for a number of respiration-related genes including *ROX1,* which acts with the general repressor complex Ssn6–Tup1 to repress transcription of hypoxic genes. However, in the absence of heme, Hap1 forms a complex with Hsp82 and Ydj1 to repress *Rox1* transcription [225, 226, 240]

Hap1-dependent regulation. Zhang and Guarente showed that Hap1 is a nuclear factor but that, in the absence of heme, it is sequestered in a high molecular weight cytoplasmic complex [239]. More recent studies by Zhang et al. [226] have suggested that in the absence of heme, Hap1 forms a higher order complex with four other proteins including the heat shock proteins Hsp82 and Ydj1. Formation of this complex correlates with the repression of *ROX1* [240].

Homologs of *HAP* genes have been found in other yeast systems. In *S. pombe* deletion of a *HAP2* homolog, *phb2*, appears to affect mitochondrial function, but in *Kluyveromyces lactis,* deletion of *HAP2* and *HAP3* homologs had no significant effect on its ability to grow on respiratory substrates [196].

8.2.2 *Rox1*

The *ROX1* gene encodes a trans-acting product (Rox1), which is involved in regulating heme-repressed and heme-induced genes in *S. cerevisiae* [241]. Rox1 is a DNA-binding repressor of respiration-related (hypoxic) enzymes. In the presence of oxygen and heme, Rox1 represses transcription of several genes that are important for growth under oxygen-limited conditions (Fig. 11). These hypoxic genes include the transcriptional activator *ANB1*, the cytochrome isoforms *CYC7* and *COX5b,* a gene for heme biosynthesis, *HEM13,* a gene for an oxygen-dependent step in the biosynthesis of ergosterol, *ERG11*, and a mitochondrial ADP/ATP translocator, *AAC3* [242]. Together, Hap1 and Rox1 form a

regulatory pathway in the repression of *HEM13* transcription in *S. cerevisiae* [234]. Hap1 activates transcription of *HEM13* in the absence of heme. The repression of *HEM13* transcription caused by heme involves both Hap1 and Rox1. A mutation in either gene results in derepression of *HEM13* expression.

The major cytochrome P450 in the yeast *S. cerevisiae*, lanosterol 14-alpha-demethylase (Erg11), catalyzes an essential reaction in the biosynthesis of ergosterol, the predominant sterol of yeast. Carbon source, oxygen, heme and the growth state of the culture affect expression of this protein. *ERG11* mRNA increases during growth on glucose in the presence of heme, during oxygen-limiting growth conditions, and during anaerobic growth. Two upstream activating sequences, UAS1 and UAS2, and an upstream repressor element, URS1, have been identified in the *ERG11* promoter. Hap1 activates transcription from UAS1 but not from UAS2. Sequences resembling *ERG11* UAS2 have been identified in numerous other oxygen-regulated genes. Repression of ERG11 expression depends upon the Rox1 repressor and additional repressor(s). These data indicate that *ERG11* is a member of the hypoxic gene family [243]. Furthermore, the gene coding for NADPH-cytochrome P450 reductase (*CPR1*), another component in this P450 system, appears to be coordinately regulated with *ERG11*.

8.2.3 *Hap2/3/4/5*

The principal transcriptional activator for production of respiratory proteins in *S. cerevisiae* is Hap2/3/4/5, which acts in concert with Hap1 to induce production of the mitochondrial machinery in a carbon-source dependent manner (Fig. 12). Hap2/3/4/5 is a highly conserved heteromeric complex that binds to a consensus TNATTGGT sequence called the CCAAT box, which is found in the 5′-UTR of many respiration-related genes. It activates genes in response to the presence of oxygen or non-fermentable carbon sources. A 60 amino acid core region of Hap2 is sufficient for DNA binding of the Hap2/3/4/5 complex. Hap3 and Hap5 contain a histone fold motif, which promotes protein–protein or protein–DNA interactions in many proteins. Hap4 is mainly responsible for transcriptional activation [196]. The *HAP4* gene is regulated transcriptionally by a

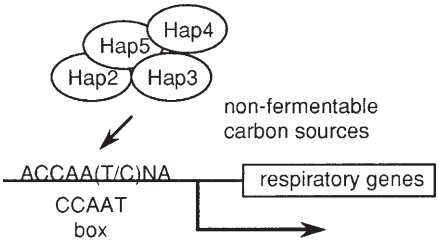

Fig. 12. Transcriptional activation by the Hap2/3/4/5 complex. In the presence of non-fermentable carbon sources, the Hap2/3/4/5 complex activates transcription of various respiratory genes [196]

carbon source, suggesting that it encodes a regulatory subunit of the bound complex [244].

The essential core protein of the transcriptional activator Hap2 appears to be stringently conserved because Olesen et al. [245] were able to isolate a homolog of Hap2 from the highly divergent fission yeast, *S. pombe*, by functional complementation of a *S. cerevisiae hap2* mutant. The *S. pombe* homolog (*S. pombe* Hap2) retains the ability to associate with Hap3 and Hap4. It contains a 60 amino acid block that is 82% identical to the *S. cerevisiae* core structure, but the remainder of the 334 amino acid protein is completely without homology to Hap2. Like *HAP2* in *S. cerevisiae*, the *S. pombe* gene is specifically involved in mitochondrial function.

8.2.4
Oxygen Regulation in Other Organisms

Researchers already recognize several differences in regulatory mechanisms among yeasts. For example, ubiquinol cytochrome *c* is subject to glucose repression in *S. cerevisiae* but in *K. lactis* it is not, even though the homologous promoter regions in the two yeasts share binding sites for several transcription factors, including Hap2/3/4/5 [246]. In another example, homologs of a mitochondrial gene of *K. lactis* and *S. cerevisiae* share a binding site for the transcription factor complex Hap2/3/4/5, but the presence of binding sites for another transcriptional binding factor, Cpf1, alters the regulatory properties of the promoter completely [247]. Indeed, when a functional homolog of the *S. cerevisiae HAP2* gene was cloned from and disrupted in *K. lactis*, inactivation of the gene had no significant effect on respiration-dependent growth. This suggested that the Hap2/3/4/5 complex has no major control over the formation of the mitochondrial respiratory system in *K. lactis* [248].

9
Ethanol Tolerance and ATPase Activity

S. cerevisiae has unique machinery that gives it a higher ethanol tolerance than *P. stipitis*. This could be attributed to its higher proton-pumping ATPase activity. *S. cerevisiae* can tolerate a loss in the ATPase activity of up to 70% while *P. stipitis* stops growing after a 40% loss. In addition, growth of *S. cerevisiae* in the presence of ethanol reduces the effect of ethanol on proton influx. This adaptation phenomenon is not observed in *P. stipitis*. Yeast cells can reoxidize ethanol so that acetic acid reduces the intracellular pH [249]. This alters the trans-membrane proton flow, which is critical for active transport and the generation of ATP. The net trans-membrane proton gradient results from the difference between proton influx (due to uncoupled diffusion and proton mediated symport) and active proton extrusion. Dissipation of the proton motive force and cytoplasmic acidification affect multiple cellular metabolic activities [250, 251]. Ethanol can influence the net trans-membrane proton flow in *S. cerevisiae* [252, 253]. However *S. cerevisiae* can tolerate ethanol concentrations of 70 to

110 g/l [254]. By comparison, *P. stipitis* stops growing when the ethanol concentration in the medium is over 30 g/l [255].

The oxygen requirement that *P. stipitis* has for growth and xylose transport could be additional factors contributing to its lower ethanol tolerance. Under aerobic conditions, more ATP is generated for transport than is necessary because of the active xylose uptake system, but a portion of the ethanol might also be oxidized to acetic acid, thereby lowering the intracellular pH and reducing the trans-membrane proton gradient. *P. stipitis* engineered to grow under strictly anaerobic conditions can form and adapt to up to 6.5% ethanol from glucose [256], so formation of acetic acid probably plays a critical role in limiting ethanol tolerance under low aeration conditions.

Ethanol inhibition in *P. stipitis* is associated with a decreased plasma membrane ATPase activity. Meyrial et al. [257] reported that glucose-grown *P. stipitis* displays threefold higher plasma membrane ATPase activity than xylose-grown cells. In a continuous culture study, they found a new mechanism of ethanol inhibition in *P. stipitis* [258]. For both xylose- and glucose-grown cells under fermentative conditions, the proton influx rate was not affected by ethanol concentrations ranging from zero to 30 g/l. However, when ethanol concentrations were higher than 60 g/l, proton influx rates increased. Xylose-grown cells showed higher ethanol susceptibility and a higher proton influx rate than glucose-grown cells when researchers added 10 g/l ethanol to the growth medium. Glucose activated both ATP hydrolysis and proton-pumping activities of the H^+-ATPase while ethanol caused an uncoupling between the ATP hydrolysis and the proton-pumping activities. The uncoupling could be the cause of ethanol-induced growth inhibition of glucose-grown *P. stipitis*. These observations provide a new line of evidence supporting the notion that *P. stipitis* relies on different energy-generating pathways to utilize glucose and xylose.

10
Genetic Development of Xylose-Fermenting Organisms

Genetic development has proceeded by selecting improved mutants, creating hybrids, and genetic engineering for new traits. In the case of *Saccharomyces*, genes for xylose metabolism have been taken from *Pichia stipitis*, and for *P. stipitis,* a gene enabling anaerobic growth has been taken from *Saccharomyces*. Much, however, remains to be done.

10.1
Selection of Glucose-Derepressed Mutants

Several research groups have looked for improved mutants of xylose-fermenting yeasts. One approach has been to use 2-deoxyglucose (2-DOG) to obtain carbon catabolite derepressed strains [259, 260]. Strains resistant to 2-DOG are better able to ferment other sugars. Because 2-DOG cannot be metabolized beyond phosphorylation, wild-type cells take it up and phosphorylate it to 2-DOG-6-phosphate, which then represses pathways that metabolize other sugars such as maltose, sucrose or galactose.

In *K. lactis*, mutants devoid of hexokinase showed relief from carbon catabolite repression of several enzymes [261]. Alamae and Simisker screened 2-DOG-resistant colonies of the methylotrophic yeast, *Pichia pinus*, for the formation of alcohol oxidase activity in the presence of glucose. All of the mutants that they obtained grew poorly on D-xylose compared to the wild type, whereas growth on L-arabinose was similar to the wild type [262]. Wedlock et al. selected mutants of *P. tannophilus* with decreased ability to utilize D-glucose as the sole carbon source by selecting for resistance to 2-DOG. Enzyme studies confirmed that these strains are defective in hexose-phosphorylating enzymes and are unable to phosphorylate D-glucose to D-glucose-6-phosphate [263].

Pardo et al. [264] obtained mutants of *P. stipitis* defective in carbon catabolite repression by selecting for resistance to 2-DOG as a sole carbon source. Other researchers have used 2-DOG in the presence of a glucose-repressible carbon source. One study examined both methods and screened mutant strains for improved use of xylose in the presence of glucose [265]. *P. stipitis* mutants selected for growth on D-xylose in the presence of 2-DOG used xylose from a 1:1 glucose/xylose mixture more rapidly than did their parents. One of these, FPL-DX26, completely consumed xylose in the presence of glucose and produced 33 g/l ethanol in 45 h from 80 g/l of this sugar mixture. Mutants selected for growth on 2-DOG alone did not show significant improvement.

10.2 Expression of *Pichia* Genes in *Saccharomyces*

The metabolic engineering of xylose fermentation in *S. cerevisiae* has been progressively more successful. Although several genes for xylose metabolism from *P. stipitis* have been expressed in *S. cerevisiae*, they have not had all the intended effects on xylose metabolism. Genes for *P. stipitis* xylose reductase (*XYL1*) [99, 103, 266], *XYL1* plus xylitol dehydrogenase (*XYL2)* [50, 51, 267, 268], transketolase (*TKT*) plus *XYL1* and *XYL2* [269], or *TKT* plus transaldolase (*TAL*) and *XYL1* and *XYL2* [270] have been expressed in *S. cerevisiae* in order to impart xylose fermentation. The introduction of *XYL1* from *P. stipitis* did not enable *S. cerevisiae to* grow on or produce ethanol from xylose. Heterologous production of *XYL1* alone does enable it to make xylitol from xylose, as long as a supplemental carbon source is provided. Galactose is particularly useful because it does not compete with xylose for transport [50]. The presence of both *XYL1* and *XYL2* enables *S. cerevisiae* to grow on xylose [51, 52], but it is necessary to also overexpress the gene for xylulokinase, *XKS1*, in order to obtain significant growth or ethanol production on xylose [53]. A *S. cerevisiae* fusion strain containing *PsXYL1*, *PsXYL2* and *XKS1* showed higher fermentative capacity on glucose and xylose [271]. Two other genes, the transketolase and transaldolase genes from *P. stipitis*, have been cloned and introduced into *S. cerevisiae* to improve xylulose conversion. However, they do not show significant effects in the recombinant *S. cerevisiae* strains unless *XYL1* and *XYL2* are overexpressed at the same time [272]. In some genetic backgrounds, e.g. *Saccharomyces* expressing *XYL1* and *XYL2* plus xylulokinase [273], ethanol production from xylose apparently increases. Presumably, overexpression of the native *Saccharomyces XKS1* increases xylose metabolism in this organism.

10.3 Development of a Genetic System for *Pichia stipitis*

The electrophoretic karyotype of *P. stipitis* reveals that it has at least six chromosomes in its genome [274]. Sporulation studies show that *P. stipitis* has a homothallic life cycle. Asci containing two hat-shaped ascospores are formed by wild-type strains on nutritionally poor media [275]. Mating can be forced by growing the cells on media containing low levels of malt extract, and stable diploids can be maintained on rich medium [276]. High mutation frequencies caused by UV irradiation of *P. stipitis* wild-type strains imply that *P. stipitis* is haploid. However, some lines of evidence suggest that homothallic diploids are prevalent [277]. Disruption studies performed in *PsADH1*, *PsADH2* and *PsLEU2* genes cloned from CBS 6054 demonstrate that transformable *ura3* strains derived from CBS 6054 are diploids [187, 278]. Disruption of these genes is more difficult than would be expected if these strains derived from CBS 6054 were haploids. In addition to homozygous integration, numerous gene conversion, heterozygous integration, and random integration events are detected. It is necessary to screen numerous transformants in order to find one that is site-specific. Taken together, these results indicate that at least some of the parental strains (or resulting disruptants) are diploids.

The frequency of homologous recombination at any given locus in *P. stipitis* seems to be lower than in *S. cerevisiae* and varies somewhat from locus to locus within the genome. In the case of the *PsADH1* gene disruption, only one of 17 Ura^+ transformants was a homozygous integrant at the corresponding genomic locus.

To genetically manipulate *P. stipitis*, it is essential to have selectable markers to allow transformed cells to grow while preventing the growth of non-transformed cells. Drug resistance markers, for example, kanamycin resistance, has been developed in *P. stipitis* [279]. The transformation frequencies obtained with this system, however, are very low. Moreover, using drug resistance as a selectable marker requires adding the drug in the fermentation medium to maintain the vector DNA in the cells. This is prohibitively expensive and unpractical in a large-scale setup. Auxotrophic selection markers can avoid this problem. They have been used commonly in other yeasts. The most popular markers are genes encoding amino acid biosynthetic enzymes and transformation events are easily selected by omitting the amino acids from the medium.

The first step in developing a high-frequency transformation system was to clone the *P. stipitis URA3* gene through cross hybridization with its *S. cerevisiae* homolog [280]. This approach took advantage of the powerful positive selection system for *ura3* mutants afforded by 5′-fluoroorotic acid [281]. In order to increase the efficiency of transformation and the capacity to recover constructs, a native *P. stipitis* autonomous replication sequence (*ARS)* was isolated. The third part of the system is a *ura3* recipient host which was initially isolated as a spontaneous mutant (FPL-TJ26, *ura3–1*) from *P. stipitis* CBS 6054. With electroporation using circular *ARS*-bearing vectors, this system can produce 600 to 8000 transformants per μg of DNA. Linearized vectors produce up to 12,000 transformants per μg of DNA.

Fermentation studies with the initial transformable host, FPL-TJ26 (*ura3–1*), showed that it has relatively little capacity for ethanol production, so in subsequent mutagenesis studies, additional *ura3* mutants were isolated from CBS 6054 (FPL-PSU1, *ura3–2*) and from the highly fermentative mutant FPL-DX26 (FPL-UC7, *ura3–3*). All three of these recipient hosts can be transformed with *PsURA3* or with the *URA3* gene from *S. cerevisiae.* Multiple hosts give a variety of genetic backgrounds for expression, but in order to introduce more than a few genes, it is necessary to have multiple selectable markers and a mating system.

P. stipitis URA3 (*PsURA3*) was used to disrupt *P. stipitis LEU2* in *P. stipitis* FPL-UC7, a highly fermentative host. A *URA3*:lacZ "pop-out" cassette was constructed containing *PsURA3* flanked by direct repeats from segments of the lacZ reading frame. The *P. stipitis LEU2* gene (*PsLEU2*) was cloned from a *P. stipitis* CBS 6054 genomic library through homology to *S. cerevisiae LEU2*, and a disruption cassette was constructed by replacing the *PsLEU2* reading sequence with the *PsURA3*:lacZ cassette following the approaches of Alani et al. [282] and Toh-e [283]. FPL-UC7 (*ura3–3*) was transformed with the disruption cassette, and a site-specific integrant was identified by selecting for the Leu^- Ura^+ phenotype. The *ura3* marker was recovered from this strain by plating cells onto 5′-FOA and screening for spontaneous *URA3* deletion mutants. Excision of the flanked *PsURA3* gene resulted in the Leu^- Ura^- phenotype. The double auxotrophs are stable and can be transformed at a high frequency by *PsLEU2* or *PsURA3* carried on ARS-based plasmids [278].

10.4 Anaerobic Growth

Most Crabtree-negative yeasts rely on respiro-fermentative metabolism to support cell growth [284]. Molecular oxygen in yeasts acts as a terminal electron acceptor. It is required for biosynthesis of membrane sterols [285] and it is involved in sugar uptake [57]. Under strictly anaerobic conditions, *P. stipitis* can grow only for one generation and it produces low yields of ethanol [55]. The dependence of *P. stipitis* on oxygen for growth can lead to re-oxidation of ethanol. Moreover, the requirement for controlled aeration increases processes and control costs.

S. cerevisiae can grow anaerobically using energy solely generated from fermentation when essential lipids are present [286]. Nagy et al. [287] showed that *S. cerevisiae* possesses a unique form of dihydroorotate dehydrogenase (DHOdehase; EC 1.3.3.1) that confers its ability to grow anaerobically. In *S. cerevisiae*, DHOdehase is found in the cytosol where DHOdehase activity also couples to the reaction that reduces fumarate to succinate. DHOdehase, encoded by *ScURA1* [288], catalyzes a single redox reaction converting dihydroorotate to orotate in the pyrimidine biosynthesis pathway. DHOdehase of higher eukaryotes are functional components of the respiratory chains using oxygen as the ultimate [289], but not necessarily the only, electron acceptor [290].

Growth of *P. stipitis* requires oxygen, so it was of interest to know whether heterologous expression of the *ScURA1*gene could give it the capacity to grow

anaerobically. Shi and Jeffries [256] found that expression of *S. cerevisiae URA1 (ScURA1)* in *P. stipitis* enabled rapid anaerobic growth in minimal defined medium containing glucose when essential lipids were present. Initial *P. stipitis* transformants grew and produced 32 g/l ethanol from 78 g/l glucose. Cells produced ethanol even higher and faster following two anaerobic serial subcultures. Control strains without *ScURA1* were incapable of growing anaerobically and showed only limited fermentation. *P. stipitis* cells bearing *ScURA1* were viable in anaerobic xylose medium for long periods, and supplemental glucose allowed cell growth, but xylose alone could not support anaerobic growth even after serial anaerobic subculture on glucose [257]. This was perhaps not surprising because recombinant *S. cerevisiae* with the xylose assimilation genes from *P. stipitis* cannot convert xylose to ethanol under anaerobic conditions. Moreover, native *S. cerevisiae* can produce ethanol from xylulose, but requires mitochondrial activity to metabolize this sugar [291]. These findings suggest that *P. stipitis* can grow anaerobically using metabolic energy generated through fermentation but that it exhibits fundamental differences in cofactor selection and electron transport with glucose and xylose metabolism [292]. This fundamental difference might extend to other yeasts as well.

11 Future Directions

In the past 8 years, researchers have identified important genes for xylose metabolism and expressed them in *S. cerevisiae* with varying levels of success. This has given insight into the basic biochemistry and genetics of xylose fermentation in yeasts. Researchers have gained much more knowledge about xylose transport, cofactor specificity, and substrate binding of xylose assimilating enzymes. This has suggested ways to alter responses to cofactor induction. The demonstrated presence of an alternative oxidase pathway suggests that it may play an essential role in supporting xylose metabolism. Characterization of the essential fermentative genes of *P. stipitis* shows that they are modulated by a regulatory mechanism that differs significantly from that in *S. cerevisiae*. These findings suggest that changes in regulation could affect fermentation and respiration pathways.

While progress has been significant, our knowledge is still incomplete. We need to better understand the mechanisms by which xylose-fermenting yeasts accomplish ethanol production if we are to achieve increased rates of ethanol production in *S. cerevisiae, P. stipitis* or any other yeast. We need to understand how the various elements of the *P. stipitis* system work together to result in ethanol production from xylose, and we need to know how this differs from the glucose fermentation in *S. cerevisiae.* First, however, we must better understand the glucose and oxygen regulatory system in this organism. Probably the machinery will follow a pattern known for *S. cerevisiae* and other fungi. Disruption of the corresponding regulatory proteins in *P. stipitis* could elucidate their functions and might improve the fermentative properties of the organism. The development of further transformation and expression systems for *P. stipitis* will enable these studies.

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Successful Design and Development of Genetically Engineered Saccharomyces Yeasts for Effective Cofermentation of Glucose and Xylose from Cellulosic Biomass to Fuel Ethanol

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Ethanol is an effective, environmentally friendly, nonfossil, transportation biofuel that produces far less pollution than gasoline. Furthermore, ethanol can be produced from plentiful, domestically available, renewable, cellulosic biomass. However, cellulosic biomass contains two major sugars, glucose and xylose, and a major obstacle in this process is that *Saccharomyces* yeasts, traditionally used and still the only microorganisms currently used for large scale industrial production of ethanol from glucose, are unable to ferment xylose to ethanol. This makes the use of these safest, most effective *Saccharomyces* yeasts for conversion of biomass to ethanol economically unfeasible. Since 1980, scientists worldwide have actively been trying to develop genetically engineered *Saccharomyces* yeasts to ferment xylose. In 1993, we achieved a historic breakthrough to succeed in the development of the first genetically engineered *Saccharomyces* yeasts that can effectively ferment both glucose and xylose to ethanol. This was accomplished by carefully redesigning the yeast metabolic pathway for fermenting xylose to ethanol, including cloning three xylose-metabolizing genes, modifying the genetic systems controlling gene expression, changing the dynamics of the carbon flow, etc. As a result, our recombinant yeasts not only can effectively ferment both glucose and xylose to ethanol when these sugars are present separately in the medium, but also can effectively coferment both glucose and xylose present in the same medium simultaneously to ethanol. This has made it possible because we have genetically engineered the *Saccharomyces* yeasts as such that they are able to overcome some of the natural barrier present in all microorganisms, such as the synthesis of the xylose metabolizing enzymes not to be affected by the presence of glucose and by the absence of xylose in the medium. This first generation of genetically engineered glucose-xylose-cofermenting *Saccharomyces* yeasts relies on the presence of a high-copy-number 2μ-based plasmid that contains the three cloned genetically modified xylose-metabolizing genes to provide the xylose-metabolizing capability. In 1995, we achieved another breakthrough by creating the super-stable genetically engineered glucose-xylose-cofermenting *Saccharomyces* yeasts which contain multiple copies of the same three xylose-metabolizing genes stably integrated on the yeast chromosome. This is another critical development which has made it possible for the genetically engineered yeasts to be effective for cofermenting glucose and xylose by continuous fermentation. It is widely believed that the successful development of the stable glucose-xylose-cofermenting *Saccharomyces* yeasts has made the biomass-to-ethanol technology a step much closer to commercialization. In this paper, we present an overview of our rationales and strategies as well as our methods and approaches that led to the ingenious design and successful development of our genetically engineered *Saccharomyces* yeasts for effective cofermentation of glucose and xylose to biofuel ethanol.

Keywords. Transportation fuel, Xylose reductase gene, Xylose dehydrogenase gene, Xylulokinase gene, 2μ-based yeast-*E. coli* high-copy-number plasmid, Glucose effect, Enzyme induction, Gene integration, Fermentation of biomass hydrolysates

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1 Introduction

Numerous studies have proven that ethanol as a transportation fuel produces far less air pollutants than gasoline, and particularly since it contributes no net carbon dioxide to the atmosphere. This environmentally friendly liquid fuel can be used directly as a neat fuel (100%) or as a blend with gasoline at various concentrations. The raw material used for the production of ethanol fuel is renewable and abundantly available domestically in most countries. Thus, the use of ethanol to supplement or replace gasoline not only reduces air pollution, ensures a cleaner environment, and eases the threat of global warming, but also reduces the dependency of many nations on imported foreign oil and protects their energy security.

Ethanol has traditionally been and is currently still produced from glucose-based food crops, such as cane sugar, corn starch, and other starch-rich grains, via fermentation of glucose present in these feedstocks by *Saccharomyces* yeasts. However, these agricultural crops are expensive and in limited supply.

More than twenty years ago, it was recognized that cellulosic biomass, including agricultural residues (such as corn stover, rice and wheat straws, and sugarcane bagasse), municipal wastes (such as yard and paper wastes), and industrial wastes (such as wastes from paper mills), is an attractive feedstock for ethanol-fuel production by fermentation because cellulosic biomass is not only renewable and available domestically in most countries but also available at very low cost and in great abundance.

However, there are problems that must be solved before such ideal feedstocks can be economically converted to ethanol. One serious problem has been that the *Saccharomyces* yeasts traditionally used and still the most effective microorganisms for fermenting glucose-based feedstocks to ethanol, are found to be unable to effectively ferment all the major sugars from cellulosic biomass to ethanol. This is because the major sugars derived from cellulosic biomass are not just glucose but also xylose with a ratio of glucose to xylose being approximately 2 or 3 to 1. It is generally agreed that unless both glucose and xylose from the cellulosic biomass can be fermented, the economics of converting cellulosic biomass to ethanol is unfavorable. However, nearly all of the fermentative Yeasts, including the *Saccharomyces*, are found to be unable to ferment xylose to ethanol or utilize the pentose sugar for growth. This is because these yeasts are missing certain enzyme(s) that are responsible for the conversion of xylose to ethanol. Thus, up to 1993, the major obstacle that prevented cellulosic biomass from being economically converted to ethanol had been the lack of safe and effective microorganisms, particularly the *Saccharomyces* yeasts, that can effectively and efficiently convert both glucose and xylose to ethanol.

In order to solve the above described problem, lots of research efforts, particularly in the period between 1970 to 1980, were devoted to searching for new yeasts, especially the *Saccharomyces* yeasts, that might be able to effectively ferment both glucose and xylose to ethanol. However, no such *Saccharomyces* yeasts were found. Nevertheless, a few other yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* were found to be able to ferment xylose to ethanol and also able to utilize the sugar for growth. Unfortunately, these naturally occurring xylose-fermenting yeasts have severe drawbacks that limit their commercial applicability [1].

Yeasts and bacteria metabolize xylose by following slightly different pathways as showing in Fig. 1. Yeasts rely on xylose reductase and xylitol dehydrogenase, but bacteria rely on xylose isomerase, to convert xylose to xylulose [2, 3]. Although the *Saccharomyces* yeasts as well as other fermentative yeasts are not able to ferment xylose, *Saccharomyces* yeasts are able to ferment xylulose to ethanol [4]. Furthermore, they are also able to ferment xylose when a bacterial xylose isomerase is present in the medium [5]. This indicates that *Saccharomyces* yeasts lack only the enzymes for the conversion of xylose to xylulose.

More than 15 years ago, nearly ten groups worldwide including our group – the then newly established Molecular Genetics Group at the Laboratory of

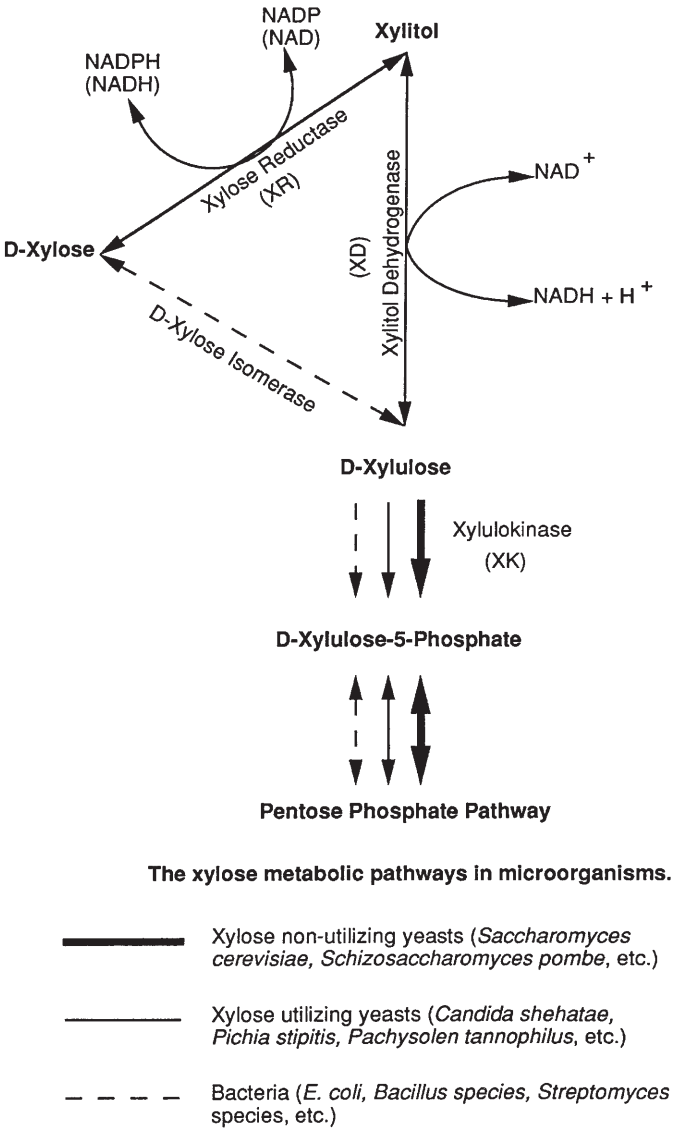


Fig. 1. The xylose metabolic pathways in various microorganisms

Renewable Resources Engineering of Purdue University – were all attempting to transfer (clone) a bacterial xylose isomerase gene into various species of *Saccharomyces* yeasts to develop genetically engineered yeasts for fermenting xylose (Fig. 1). However, all those earlier efforts failed to produce a genetically engineered yeast that could ferment xylose [6–10].

After the unsuccessful attempts to clone a xylose isomerase gene in yeast, we were one of the remaining four known groups that continued to pursue this dif-

ficult task. Although all the groups now turned to clone the xylose reductase (*XR*) and xylitol dehydrogenase (*XD*) genes from *P. stipitis* to provide the *Saccharomyces* yeasts the capability of converting xylose to xylulose [11–14], there are major differences between our approach and those of the three other groups in the design and development of the ideal yeast for fermenting the cellulosic sugars (fermentable sugars derived from cellulosic biomass) [15]. To the best of our knowledge, we are the only group even to this date that has succeeded in genetic engineering of the *Saccharomyces* yeasts for effective fermentation of xylose to ethanol. In addition, our genetically engineered yeasts can also effectively coferment simultaneously both glucose and xylose present in the same medium to ethanol [15]. The latter property proves to be extremely critical for yeast to be effective for fermenting both major cellulosic sugars to ethanol. In this paper, we report our rationales and strategies as well as our methods and approaches that led to the ingenious design and successful development of our genetically engineered yeasts for effective fermentation of glucose and xylose to ethanol.

2 Design of an Ideal Yeast for Effective Cofermentation of Glucose and Xylose Present in Cellulosic Biomass

The uniqueness of our approach is that we have taken considerations in our design not simply to engineer a yeast to be able to ferment xylose but also to be able (1) to effectively direct the carbon flow towards the production of ethanol rather than the production of byproducts such as xylitol, (2) to effectively coferment both glucose and xylose simultaneously so that the mixed sugars will be fermented as fast as possible, (3) to easily convert most *Saccharomyces* strains, particularly the superior glucose-fermenting industrial strains to coferment xylose in addition to glucose, and (4) to use rich medium for growth and fermentation to make the engineered yeast grow and ferment sugars faster and to solve the potential waste problems by recycling used yeast cells for the production of crude yeast extracts for culturing new yeast cells. Furthermore, the final genetically engineered yeasts should be stable and able to use either batch or continuous process for ethanol production without requiring the use of any special chemicals functioning as the selection pressure to maintain the cloned genes.

In order to accomplish all those described above, we believed that we needed not only to clone a xylose reductase gene (*XR*) and a xylitol dehydrogenase gene (*XD*) but also to clone a third gene, the xylulokinase gene (*XK*), even though all the *Saccharomyces* yeasts do contain a functional *XK*. Furthermore, we also needed to replace the signal sequences that controls the expression of the three cloned genes with the sequences that control the expression of yeast glycolytic genes. We expected that an engineered *Saccharomyces* yeast containing sufficient copies of the above described three genes should be able not only to effectively ferment xylose but also to effectively coferment both glucose and xylose present in the same medium.

3
Construction of Recombinant Plasmids Containing XR-XD-XK Three-Gene Cassette Capable of Transforming Saccharomyces Yeasts Able to Effectively Coferment Glucose and Xylose

According to our plan, we first synthesized the promoterless structural genes of *XR* [16] and *XD* [17] from *Pichia stipitis* chromosomal DNA and the *XK* gene [18, 19] from *S. cerevisiae* DNA by PCR techniques. The promoterless structural genes of *XD* and *XK* were then fused to the promoter of *S. cerevisiae* pyruvate kinase gene [20] by the technique of site specific mutagenesis and the resulting recombinant genes were designated as *KD* and *KK*. The promoterless structural gene of *XR* was fused to the promoter of *S. cerevisiae* alcohol dehydrogenase gene [21] by the same technique and the resulting fusion was designated as *AR* or *A*R* . The 5′ control region of *AR* consisted of the original 5′ noncoding sequence of *P. stipitis XR* from –1 to –50, followed by the *S. cerevisiae ADC1* 5′ control region (–14 to –1500 noncoding sequence) (*ADC1* promoter fragment cloned on pMA56) [22]. Because it was uncertain what effect the nucleotide sequence of –1 to –50 from *P. stipitis XR* might have on the expression of *XR* in *Saccharomyces* yeasts, the *A*R* gene was also constructed, in which the 5′ control region of *XR* was totally replaced by the intact 5′ control region of the *ADC1* gene (–1 to –1500 noncoding sequence) [21].

Since we might need to clone the three xylose-metabolizing genes (referred to below as the XYL genes) on different plasmids, for the convenience of the subsequent work we first cloned the three XYL genes on the *E. coli* pBluescript KS (–) [23] (referred to below as pKS) in such a way that the three genes would form a cassette which could be excised from the pKS plasmid together as a fragment by a single digestion with restriction enzyme Xho I. As a result, the three-gene cassette could be easily inserted together into various yeast-*E. coli* shuttle plasmids for simultaneous expression of all three genes in yeasts. Totally four such plasmids were constructed and they were designated as pKS-KK-A*R-KD-1, pKS-KK-A*R-KD-2, pKS-KK-AR-KD-3, and pKS-KK-AR-KD-4 (referred to below as pKRD-1,-2,-3, and –4, respectively) [15]. The four plasmids were different only in their *AR* gene orientation and in the promoter of the *AR* gene described above. Two such plasmids are shown in Fig. 2.

Although our goal was eventually to integrate sufficient copies of the XYL gene cassette into the yeast chromosome, we planned first to clone the XYL gene cassette into a high copy number 2μ-based yeast – *E. coli* shuttle plasmid. This would allow us to test our hypothesis, particularly whether the cloning of the three genes would make it possible to convert many *Saccharomyces* yeasts being unable to ferment xylose to recombinant yeasts that are not only able to effectively ferment xylose, but also able to simultaneously coferment both glucose and xylose to ethanol effectively.

We chose first to clone the XhoI restriction fragments containing the XYL gene cassette from pKRD-1,-2,-3,-4 into the SalI site of the 2μ-based pUCKm10 yeast -*E. coli* shuttle plasmid, resulting in the construction of pLNH-31,-32,-33, and –34 (Fig. 3) [15]. These plasmids were collectively designated as the pLNH

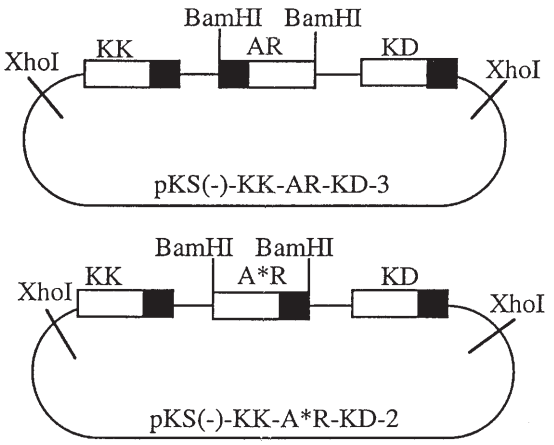
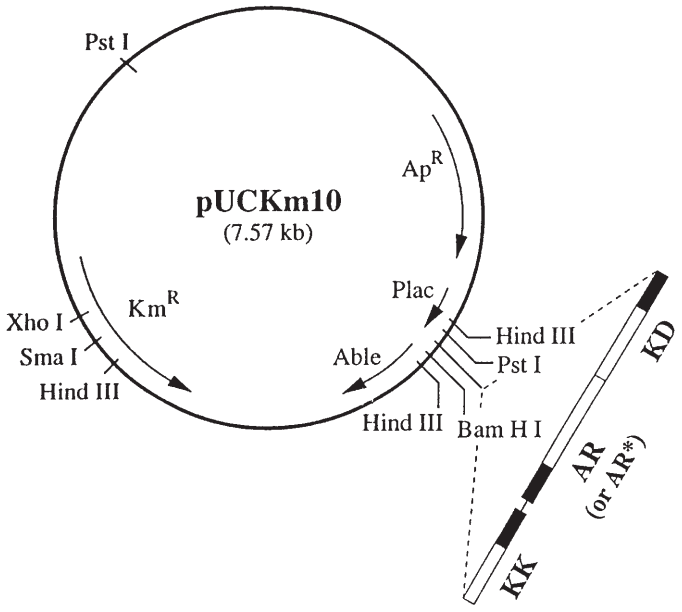


Fig. 2. The *E. coli* plasmids, pKRD-2 and pKRD-3, containing the three xylose-metabolizing gene cassette, KK-AR (or A*R)-KD



pLNH31, pLNH32, pLNH33, or pLNH34

Fig. 3. The yeast-*E. coli* shuttle plasmids containing the three xylose-metabolizing gene cassette, KK-AR (or A*R)-KD) [15]

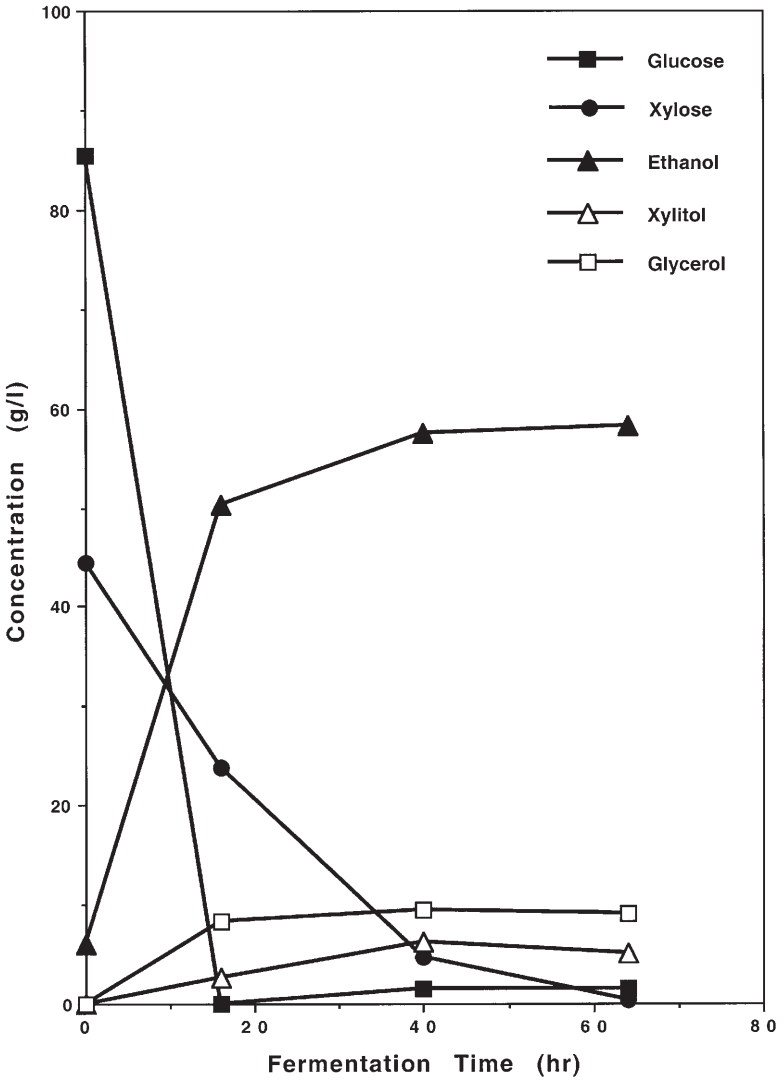


Fig. 4. Fermentation of glucose and xylose by genetically engineered *Saccharomyces* yeast strain 1400 (pLNH32) [15]

plasmids. These four plasmids were used to transform various *Saccharomyces* yeasts. The yeast transformants were initially selected by their resistance to certain levels of antibiotic geneticin and by their ability to form a halo on the ampicillin test plate [15]. Yeast transformants containing any one of the four pLNH plasmids can effectively coferment both glucose and xylose to ethanol as shown in Figs. 4 and 5. On the contrary, the parent yeast that does not contain one of the pLNH plasmids can only ferment glucose but not xylose to ethanol as shown in Fig. 6.

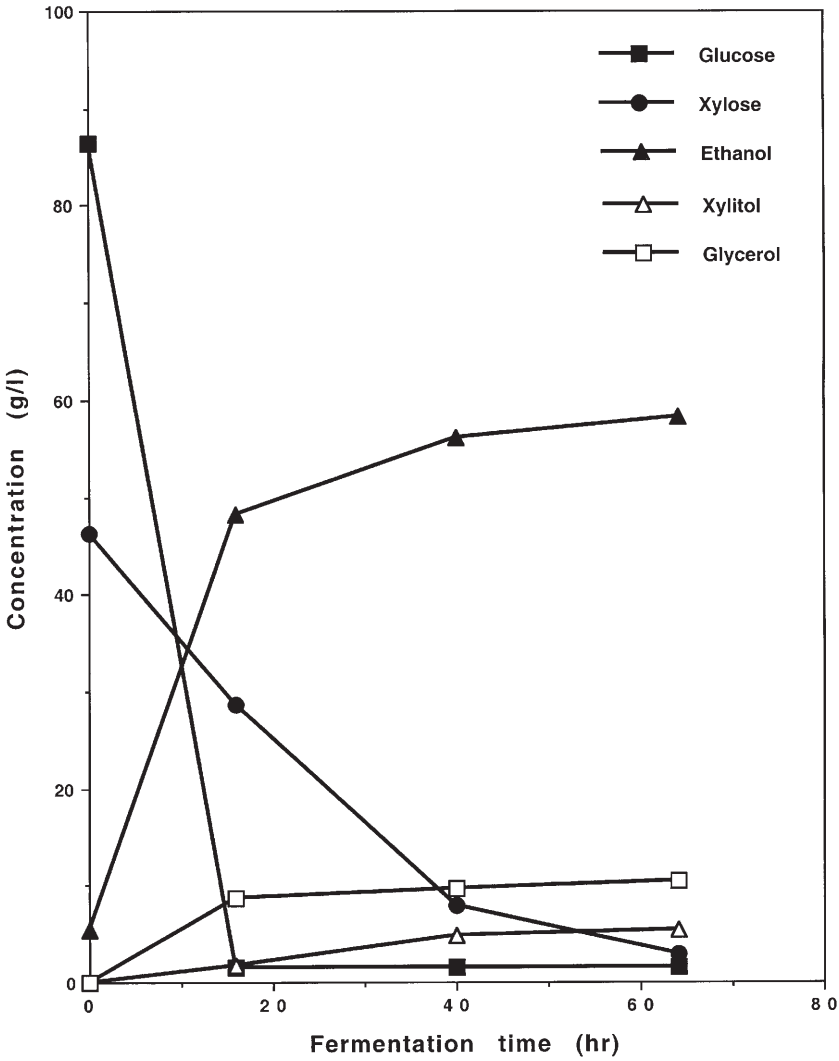


Fig. 5. Fermentation of glucose and xylose by genetically engineered *Saccharomyces* yeast strain 1400 (pLNH33)

In searching for an ideal selection system to maintain the xylose-fermenting capability of the plasmid-mediated transformants such as 1400 (pLNH32) or 1400 (pLNH33) without relying on expensive or toxic chemicals such as antibiotics, we discovered that most *Saccharomyces* yeasts are unable to grow on rich medium such as YEP (1% yeast extract, 2% peptone) without the presence of a carbon source (Fig. 7). This allows the genetically engineered yeasts to maintain their plasmids in YEPX (YEP with 2% xylose) medium without the use of antibiotic geneticin [15]. This discovery has made it possible for our en-

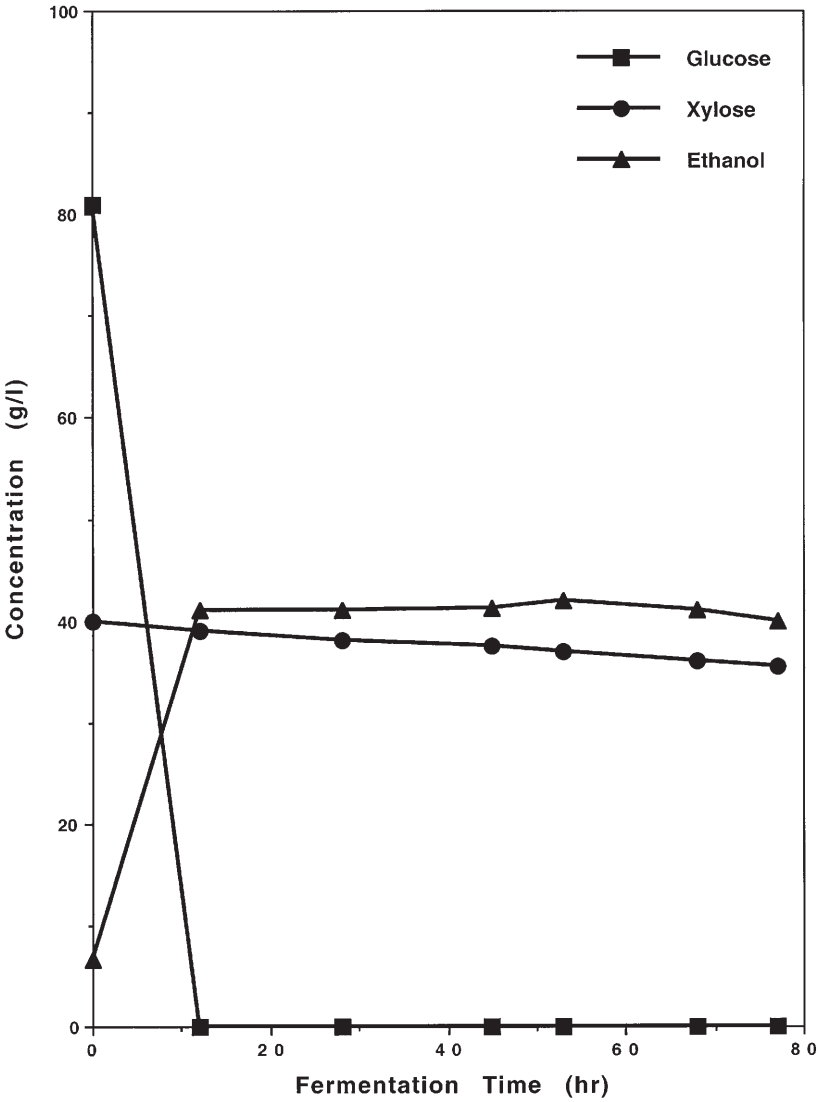


Fig. 6. Fermentation of glucose and xylose by the unengineered parent *Saccharomyces* yeast strain 1400 [15]

gineered yeasts to grow in rich medium (YEPX) without losing their xylose-fermenting capability. Since the pLNH plasmids are high-copy-number plasmids, this also allows our engineered *Saccharomyces* yeasts cultured in YEPX during early stage(s) of growth to be cultured in YEPD for the last stage of growth before fermentation to take place as described in the captions of Figs. 4 and 5.

We also found that each plasmid can transform the same yeast strains to coferment glucose and xylose with approximately the same efficiencies. For ex-

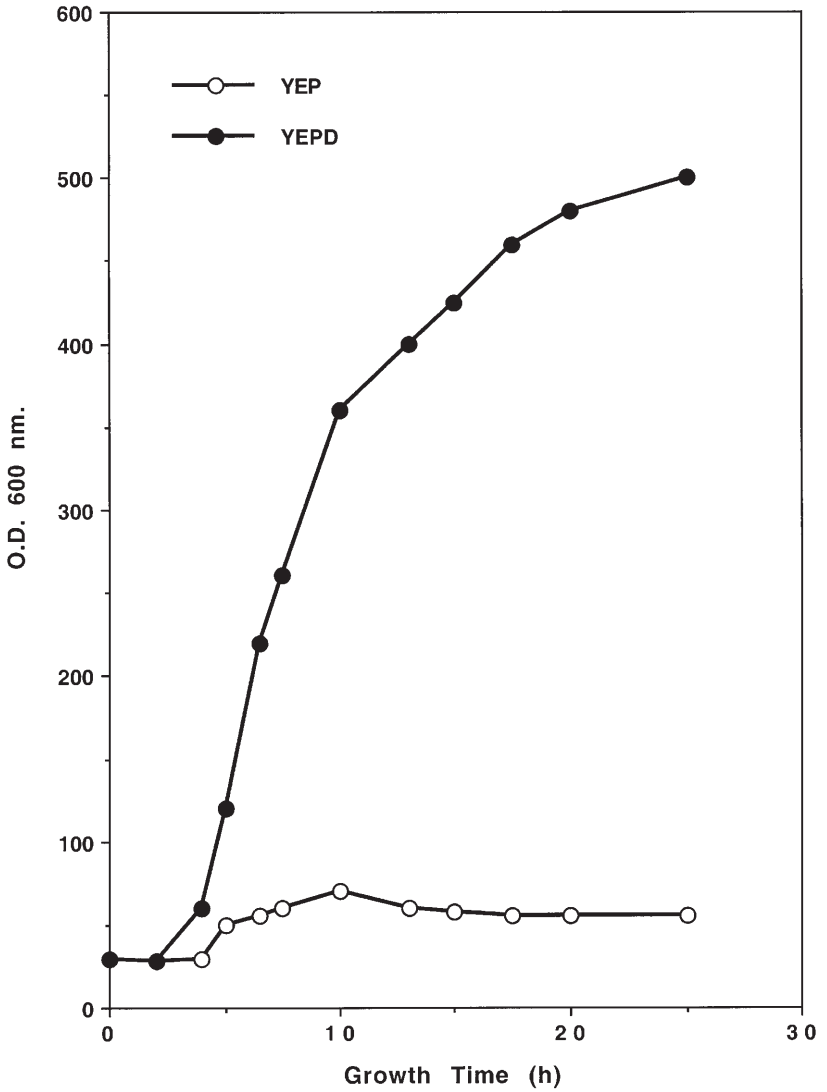


Fig. 7. Culturing of yeast *Saccharomyces cerevisiae* strain AH22 in rich medium YEP (yeast extract and peptone) with a carbon source (Glucose) or YEP alone without a carbon source (glucose) [15]

ample, the difference in efficiency between 1400 (pLNH31), 1400 (pLNH32), 1400 (pLNH33), and 1400 (pLNH34) in cofermenting glucose and xylose to ethanol is not more than 5–10% as shown in Figs. 4 and 5. This indicates that both the orientation of *AR, KD,* and *KK* on the plasmid and the promoter of *AR* or *A*R* do not affect much the efficiency of the yeast transformants in cofermenting glucose and xylose.

Table 1. Comparison of the growth of recombinant *Saccharomyces* and the parent yeast in glucose and xylose media. The units used are Klett Units, i.e. the optical density units measured by a Klett-Surnmerson photoelectric colorimeter

Strains	Medium	Time (h)						
		0	2	4	6	8	11	15
1400 (pLNH33)	YEPD[a]	21	22	44	120	289	455	472
	YEPX[b]	18	21	37	68	122	500	550
1400 (LNH-ST)	YEPD	17	19	35	87	205	400	425
	YEPX	17	18	30	59	116	482	520
Parent Yeast	YEPD	17	17	39	120	290	433	460
	YEPX	54	66	72	75	75	79	79

[a] 1% yeast extract, 2% peptone, 2% glucose.
[b] Same as [a] except 2% xylose instead of glucose.

By testing our 1400 (pLNH32) or 1400 (pLNH33), several groups have confirmed that our genetically engineered yeasts can effectively coferment glucose and xylose simultaneously as we reported above [24,25]. In particular, Moniruzzaman et al. [24] have shown that our engineered yeast ferments both glucose and xylose effectively under anaerobic conditions in a well controlled fermenter. Since our fermentations were carried out in shaker flasks sealed with Saran Wrap and limited amounts of oxygen were introduced to the fermentation flasks during sampling and possibly also during fermentation, the results of Moniruzzaman et al. demonstrate that our engineered yeast does not require the presence of oxygen in order to ferment xylose.

In addition, our genetically engineered yeasts can also very efficiently utilize xylose for aerobic growth as shown in Table 1. Furthermore, as we expected, the pLNH plasmids can transform most of the *Saccharomyces* yeasts to be able to effectively coferment glucose and xylose to ethanol. However, it may require adjusting the level of geneticin to boost the copy number of the pLNH plasmid in some of the *Saccharomyces* strains in order to achieve the optimal results in cofermenting glucose and xylose as shown in Fig. 8. Nevertheless, this does not affect the development of stable glucose-xylose-cofermenting strains containing multiple copies of the integrated XYL gene cassettes in the yeast chromosome as described below.

4 Importance of Cloning the Xylulokinase Gene

One of the major differences between our genetically engineered *Saccharomyces* yeasts and those reported by others (Kotter et al. [11], Walfridsson et al. [12] and Tantirungkij et al. [13] is that in addition to cloning the *XR* and *XD* genes, we also cloned the *XK* genes, even though nearly all *Saccharomyces* yeasts have an active xylulokinase gene and produce an active xylulokinase enzyme in the presence of xylose. As a result, our genetically engineered yeast can effec-

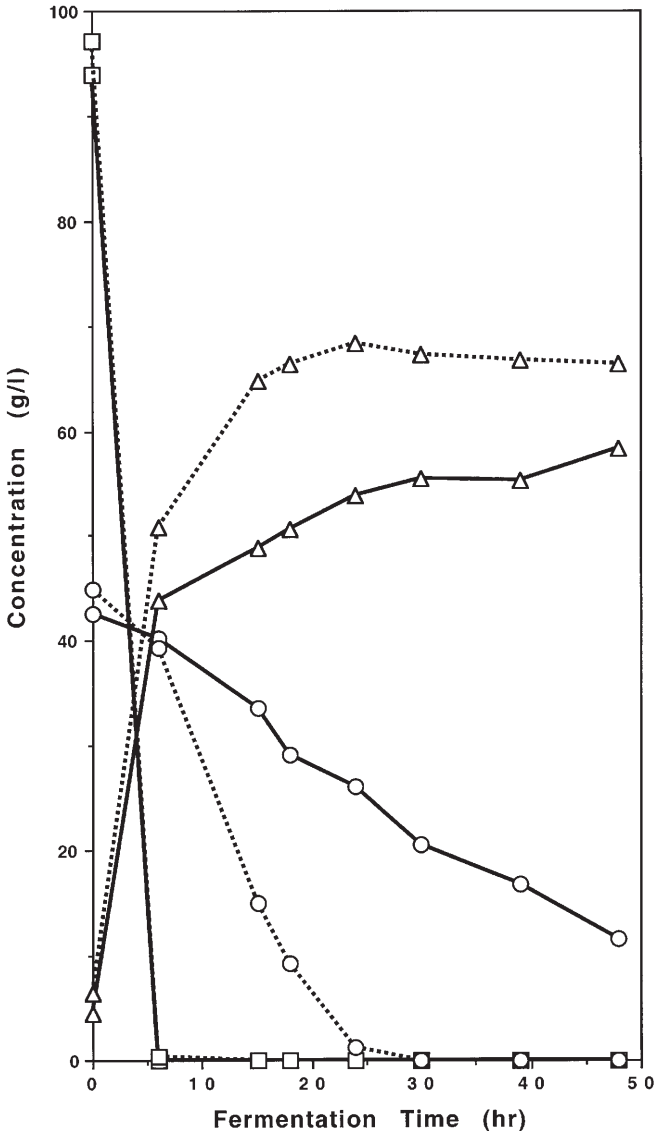


Fig. 8. Fermentation of glucose and xylose by 259A(pLNH32) in the presence (100 µg/ml) (*dotted line*) or absence of geneticin (*solid line*). Symbols: *square* glucose; *circle* xylose; *triangle* ethanol

tively utilize xylose for growth and also can effectively ferment xylose to ethanol. On the contrary, those developed by Kotter et al. [11] and Walfridsson et al. [12] are neither able to utilize xylose for growth nor able to ferment xylose to ethanol. Although Tantirungkij et al. [13] reported that their engineered yeast can grow with xylose as the sole carbon source, their yeast still cannot ferment

xylose to ethanol. We believed that the cloning and overexpression of the xylulokinase gene is extremely important in order to make yeasts ferment xylose effectively, particularly to ferment both glucose and xylose simultaneously when these two sugars are present in the same medium. In our view, cloning and overexpression of the xylulokinase gene contributed at least the following for making the *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose:

(1) Most *Saccharomyces* yeasts have very low levels of xylulokinase activity [26]. Cloning and overexpression of a xylulokinase gene in the *Sac-*

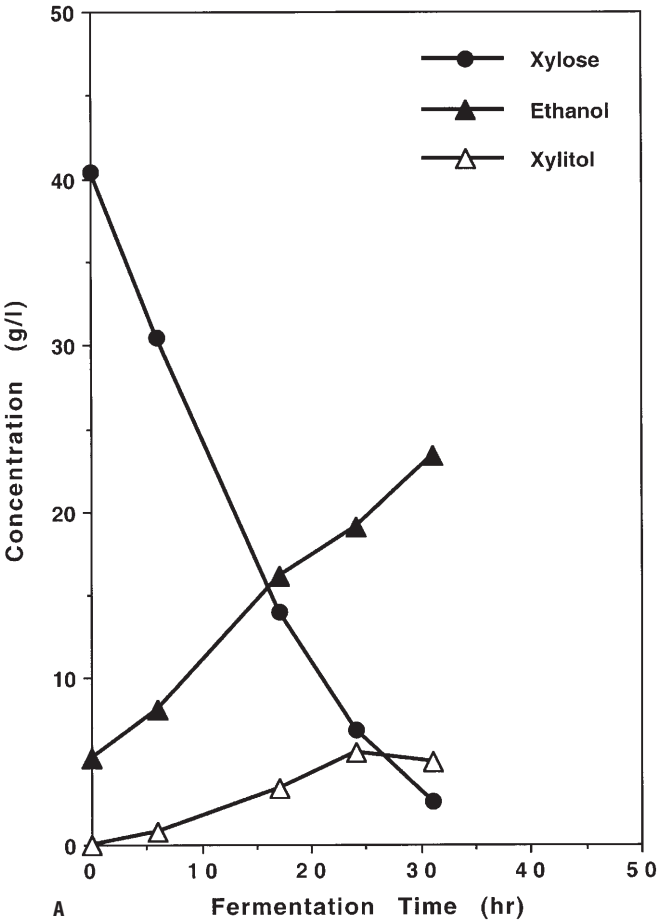


Fig. 9 A, B. Comparison of fermentation of xylose under identical conditions by **A** genetically engineered *Saccharomyces* yeast strain 1400(pLNH32) which contains the cloned and genetically modified *XR*, *XD*, and *XK* genes and by **B** 1400 (p*XR-XD*) which contains only the same cloned *XR* and *XD* genes, but not the cloned *XK* gene. These results demonstrate the importance of cloning the *XK* gene to enable the *Saccharomyces* yeasts such as 1400 (pLNH32) to ferment xylose to ethanol

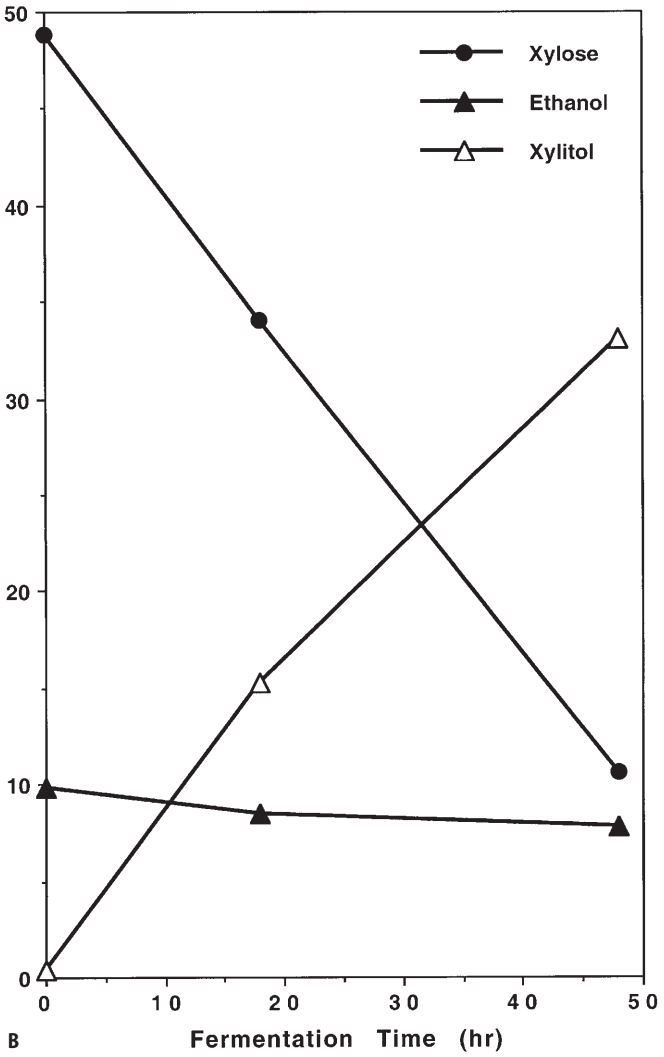


Fig. 9B (Continued)

charomyces yeasts will provide the necessary high levels of xylulokinase activity for effective metabolizing of xylose either for growth or for the production of ethanol anaerobically .

(2) The *Pichia* xylitol dehydrogenase gene chosen by us to be cloned into the *Saccharomyces* yeast is known to produce a xylitol dehydrogenase that catalyzes a reversible reaction between xylitol and xylulose as shown in Fig. 1 but favors the formation of xylitol rather than xylulose. Thus, an extremely strong xylulokinase activity will help to direct the carbon flux towards the production of ethanol rather than the formation of byproduct xylitol.

(3) According to our plan, we must alter the genetic signals controlling the expression of any genes in yeasts that will be inhibited by the presence of glucose (catabolic repression) and also require the presence of xylose for induction. The promoter of the yeast xylulokinase gene falls into these categories and therefore we must replace it with another promoter whose function will not be affected by the presence of glucose or by the absence of xylose. Thus, even a yeast containing a very effective xylulokinase gene and producing high levels of xylulokinase still needs to replace its promoter with a glycolytic promoter.

To demonstrate the effect of cloning *XK* on metabolizing xylose by the *Saccharomyces* yeast, we constructed a new plasmid pUCKm10-XR-XD which is identical with pLNH33 except without the cloned *XK* gene. *Saccharomyces* strain 1400 transformants containing pUCKm10-XR-XD utilize xylose for growth at much slower rates, less than one 15th of the rate of the same yeast strain containing pLNH33. Furthermore, the genetically engineered 1400 yeast containing pUCKm10-XR-XD, designated 1400(pXR-XD), only can convert xylose to xylitol, but not ethanol as compared in Fig. 9 A, B.

5
The Importance of Making the Genetically Engineered Yeasts Able to Coferment Glucose and Xylose

Nearly all naturally occurring microorganisms including the *Saccharomyces* yeasts, only metabolize glucose when a mixture of sugars, such as glucose and xylose, are present in their media. Furthermore, microorganisms also require to induce new enzymes to use other specific sugars, for example xylose, after glucose is depleted from their media. Thus, there usually is a long lag period after glucose has been depleted before the second sugar can be actively used by a microorganism and this phenomenon is generally known as the "glucose effect". In our view, the time for microorganisms, including yeasts, to convert a feedstock containing mixed sugars to ethanol would be much shortened if the microorganisms could be made free from the glucose effect and also not require the presence of xylose for the induction of the xylose-specific enzymes. We believed that this could be made by replacing the genetic signals controlling gene expression present within the 5′ noncoding sequences of those genes specifically required for the metabolism of xylose. Besides the *XR* and *XD* genes, the xylulokinase gene is the third gene also subject to the control of the glucose effect and also requiring the presence of xylose for induction of the synthesis of its protein. Thus, this is the third reason mentioned above that we must also clone the xylulokinase gene even if the parent yeasts contain high levels of xylulokinase activity.

In order to prove that cloning *KD*, *AR* or *A*R*, and *KK* (*XD*, *XR*, and *XK* structural genes fused to glycolytical promoters) is extremely important for cofermentation of feedstocks (or media) containing mixtures of glucose and xylose, we compared the fermentation of a mixture of glucose and xylose by 1400 (pLNH 32) and *Pichia stipitis*. As described above, the Pichia yeast is a na-

turally occurring xylose-fermenting yeast that can ferment xylose with similar efficiency at the laboratory scale under well controlled conditions as the genetically engineered 1400 (pLNH32) except that the expression of *Pichia's XD, XR,* and *XK* genes is subjected to glucose inhibition and also requires the presence of xylose for induction. As shown in Fig. 10 A, B, when these yeasts were cultured in YEPX and used to ferment a mixture of glucose and xylose, 1400 (pLNH32) can effectively coferment both glucose and xylose to ethanol and *P. stipitis* can ferment only glucose to ethanol but not xylose. Furthermore, our engineered yeast cultured in 50 ml YEPD medium with 2 ml of YEPX pregrown cells served as the seed culture is also able to ferment a mixture of glu-

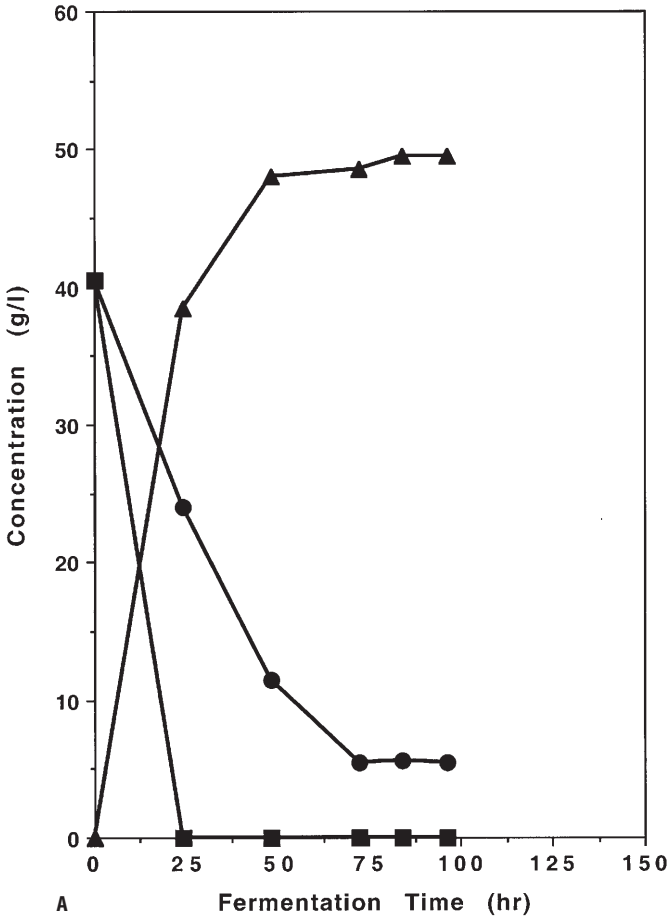


Fig. 10 A, B. Comparison of cofermentation of glucose and xylose present in the medium under identical conditions by **A** genetically engineered *Saccharomyces* yeast strain 1400 (pLNH32) and by **B** *Pichia stipitis*. These results demonstrate that our genetically engineered 1400(pLNH32) can effectively coferment glucose and xylose to ethanol but not *P. stipitis*. Symbols: *square* glucose; *circle* xylose; *triangle* ethanol

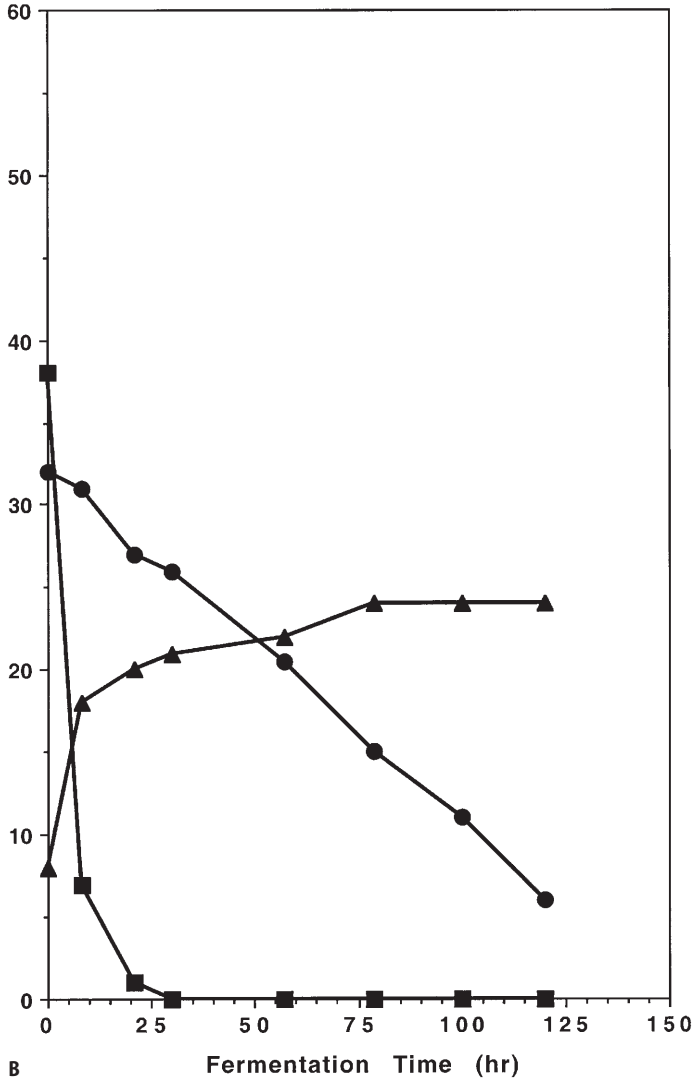


Fig. 10B. (Continued)

cose and xylose to ethanol with similar efficiency as shown in Fig. 9. Thus, our results demonstrated that it is absolutely essential to make a microorganism, for example a *Saccharomyces* yeast, able to synthesize xylose-metabolizing enzymes in the presence of glucose in order for a microorganism (yeast) to be effective in cofermenting both glucose and xylose to ethanol.

6
Effective New Integration Method for the Development of Super-stable Genetically Engineered Saccharomyces Yeasts Containing Multiple Copies of the XR-XD-XK Cassette Integrated into the Yeast Chromosome

Even though 1400(pLNH32) and related plasmid-mediated genetically engineered xylose-fermenting *Saccharomyces* yeasts are very stable and their xylose-fermenting ability can be maintained by an ideal selection mechanism, a perfect yeast for fuel-ethanol production should be completely stable without the need for using selection pressure to maintain its xylose-fermenting ability at any stage of growth or fermentation. In 1995, we successfully developed the first super-stable strain of genetically engineered glucose-xylose-cofermenting *Saccharomyces* yeast, 1400 (LNH-ST), which is completely stable without the

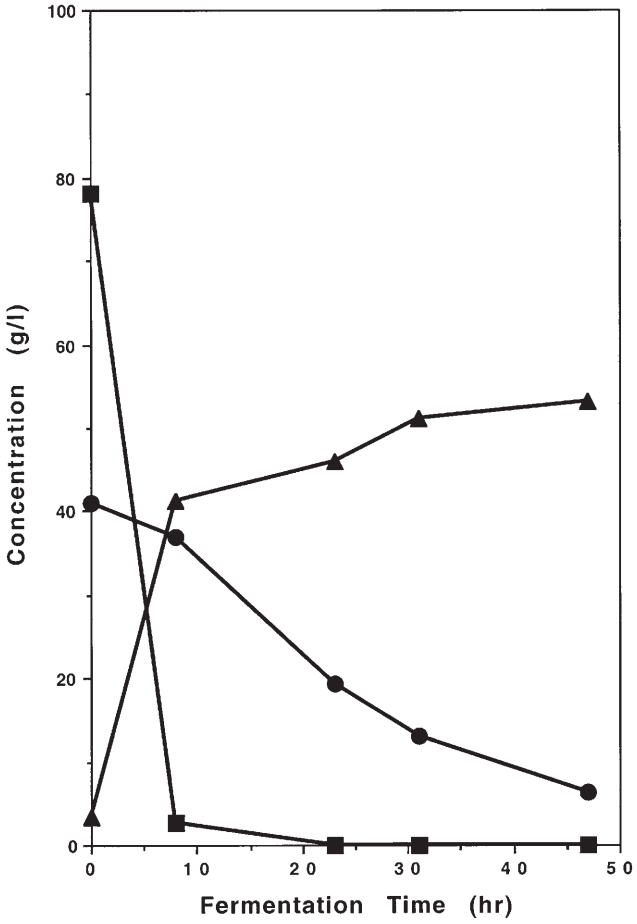


Fig. 11A. Comparison of fermentation of glucose and xylose under identical conditions by A genetically engineered *Saccharomyces* yeast strain 259A (LNH-ST)-2 and by ...

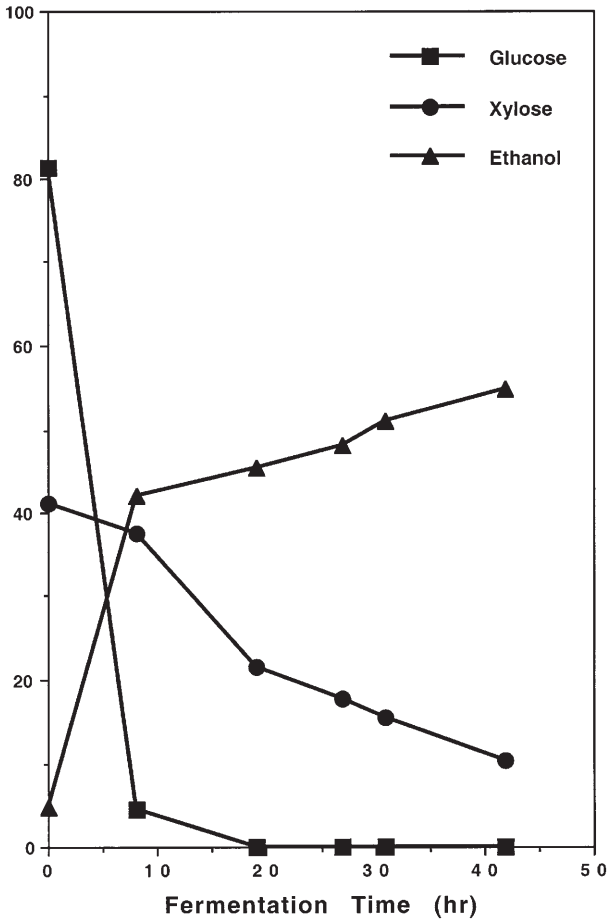


Fig. 11B. (Continued) **B** 259A (LNEI-ST)-2 after being cultured in non-selective medium (YEP + glucose) for more than 50 generations. These results demonstrate that the *Saccharomyces* yeast strain 259A (LNH-ST)-2 is stable and retain its ability to effectively ferment xylose without relying on the presence of any selection pressure. On the contrary, genetically engineered yeast 259A (pLNH32), with the xylose metabolizing genes cloned on the high copy number plasmid pLNH32, will lose its ability to ferment xylose after being cultured in the same non selective medium for even just 20 generations (data not shown). Also note that 259A (LNH-ST)-2 is one of the 259A strain containing the integrated xylose metabolizing genes that was chosen to carry out these studies but not the one that is most effective for fermenting xylose. Symbols: *square* glucose; *circle* xylose; *triangle* ethanol

need to apply any selection pressure to maintain its ability to ferment xylose to ethanol (data not shown) or utilize the sugar for growth as shown in Table 1. Furthermore, these superstable xylose-fermenting genetically engineered yeasts also coferment glucose and xylose to ethanol as effectively as or better than the plasmid-mediated recombinant yeasts such as 1400 (pLNH32) (data

not shown). These superstable genetically engineered *Saccharomyces* yeasts contain multiple copies of the same three gene cassette integrated into the host chromosome. Also our superstable xylose-fermenting yeast was developed by using a much improved new method for integrating multiple copies of genes into the yeast chromosome. This method for integrating multiple copies of gene(s) into the host chromosome is easy to accomplish and more importantly, it is also easy to control the copy-number of the integrated genes. Up to now, we have completed the development of three stable glucose-xylose-cofermenting *Saccharomyces* yeast strains 1400 (LNH-ST), 259A (LNH-ST), and 424A(LNH-ST) derived from three different parent strains of *Saccharomyces* yeasts. We are in the process of developing several more such stable glucose-xylose-cofermenting yeast strains, including strains derived from special yeasts used by industry for the production of ethanol from starch or wood hydrolysates. Figure 11 A,B demonstrate that the genetically engineered 259A (LNH-ST) cultured in non-selective medium for more than 50 generations still can ferment xylose as effective as 259A (LNH-ST) that has not been cultured in non-selective medium. The development of these stable genetically engineered xylose-fermenting *Saccharomyces* yeasts and the method of integrating multiple copies of gene(s) into the yeast chromosome have been briefly described previously [27] and the details in the development of these yeasts will be reported elsewhere.

7
Genetically Engineered Saccharomyces Yeasts Able to Coferment Glucose and Xylose Present in Crude Hydrolysates of Various Cellulosic Biomass by Batch or Continuous Process

Our genetically engineered *Saccharomyces* yeasts also can ferment glucose and xylose present in crude cellulosic hydrolysates very effectively as demonstrated in Figs. 12 – 15. The crude hydrolysates were prepared by different processes and provided to us by others. The hydrolysates were used directly for fermentation without any treatments to remove toxic inhibitors normally present in most hydrolysates. For fermentation, pregrown yeast cells were added to the hydrolysates to a cell density similar to those used for fermentation of pure sugars shown in Figs. 4 and 5. Yeast extracts were added to the hydrolysates to a final concentration of 1 % and the pH of the hydrolysates was adjusted to 7.0 prior to fermentation but without further adjustment during fermentation. However, effective fermentation of both glucose and xylose was also obtained with our genetically engineered yeasts by initially adjusting the pH of the hydrolysate to 5.5 and by supplementing only 0.2 % yeast extract for fermentation (data not shown). Independent studies were carried out by Moniruzzaman et al. to use 1400 (pLNH32) for fermenting sugars from hydrolysates of corn fiber and excellent results were also obtained [24].

The stable strain 1400 (LNH-ST) was confirmed to be able to effectively coferment glucose and xylose by a continuous process in a 9000-L pilot scale fermentor with both pure sugars and sugars from crude corn biomass hydrolysates as the feedstocks [25].

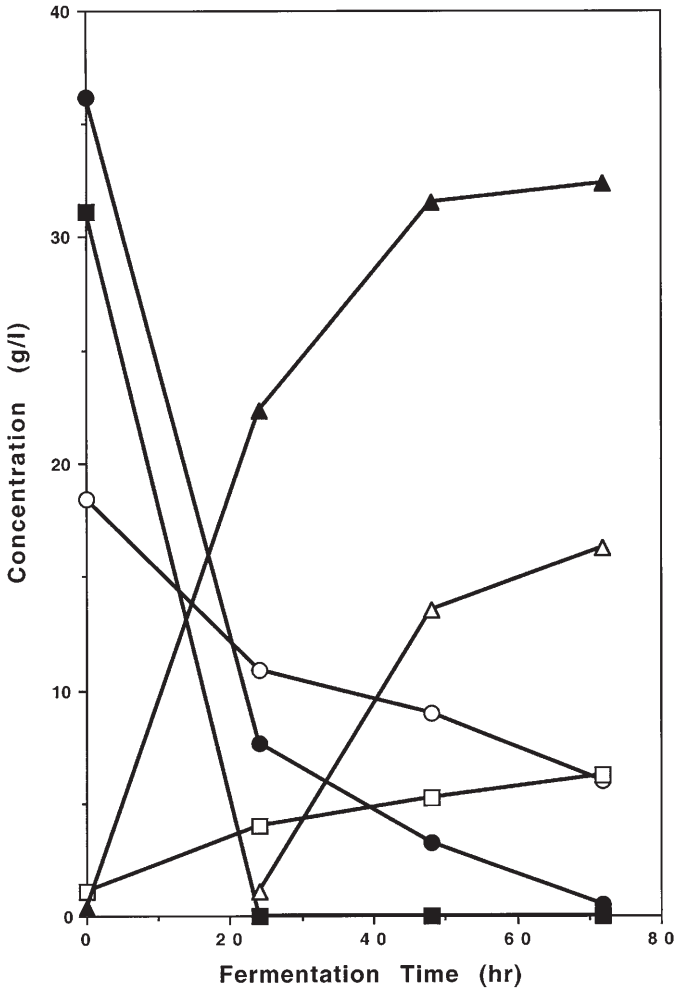


Fig. 12. Fermentation of glucose and xylose present in the crude hydrolysate of corn fiber by genetically engineered *Saccharomyces* yeast strain 1400(pLNH32). The corn fiber hydrolysate was provided by Cargill, Inc. The major sugars present in the hydrolysate are glucose, Xylose, and-arabinose. Symbols: *solid square* glucose; *solid circle* xylose; *solid triangle* ethanol; *open circle* arabinose; *open triangle* xylitol and arabitol; *open square* glycerol

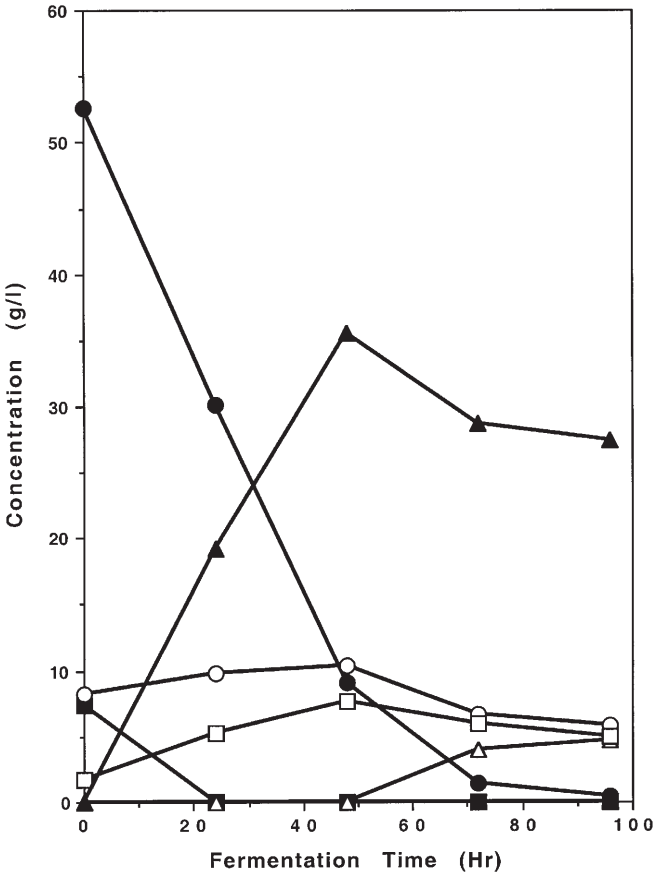


Fig. 13. Fermentation of glucose and xylose present in the crude hydrolysate of corn stover by genetically engineered *Saccharomyces* yeast strain 1400 (pLNH32). The hydrolysate was provided by Swan Biomass Company. Symbols: *solid square* glucose; *solid circle* xylose; *solid triangle* ethanol; *open circle* arabinose; *open triangle* xylitol and arabitol; *open square* glycerol

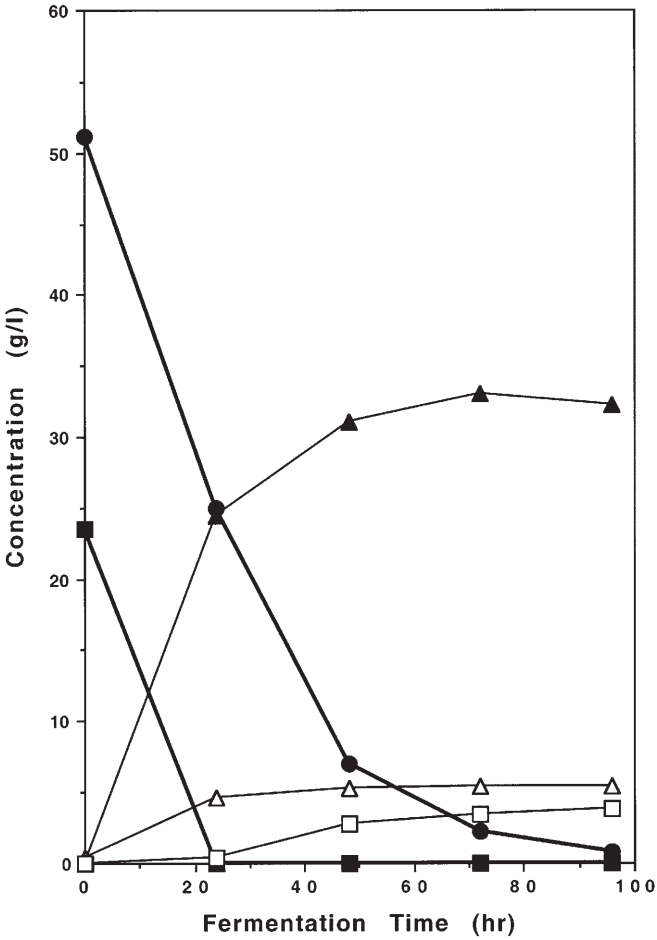


Fig. 14. Fermentation of glucose and xylose present in the crude hydrolysates of wheat straw by genetically engineered *Saccharomyces* yeast strain 1400 (pLNH32). The hydrolysate was provided by St. Lawrence Technologies Inc., Canada. Symbols: *solid square* glucose; *solid circle* xylose (90%) and small amount of galactose (10%); *solid triangle* ethanol; *open triangle* xylitol; *open square* glycerol

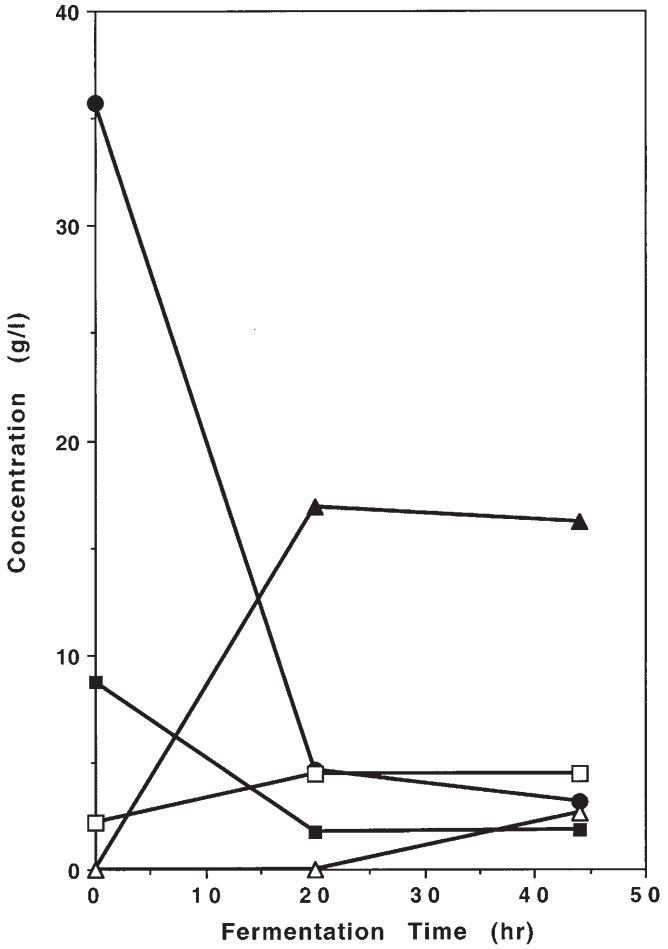


Fig. 15. Fermentation of glucose and xylose present in the crude hydrolysates of softwood by genetically engineered *Saccharomyces* yeast strain 1400 (pLNH32). The hydrolysate was provided by the Chemical Division of Tembec, Inc., Canada. Symbols: *solid square* glucose; *solid circle* mannose, xylose, and galactose; *solid triangle* ethanol; *open triangle* xylitol and arabitol; *open square* glycerol

8
Development of Genetically Engineered Saccharomyces Yeasts Able to Coferment Glucose, Xylose, and L-Arabinose

Although the major fermentable sugars from cellulosic biomass are glucose and xylose, some of them, such as corn fiber, also contain substantial amounts of L-arabinose. It is known that *Saccharomyces* yeasts are also unable to ferment L-arabinose to ethanol and to use the sugar for growth. Naturally we should try to make our engineered *Saccharomyces* yeasts also able to ferment L-arabinose to ethanol and also to use the sugar for growth, if possible.

It is known that most bacteria including *E. coli* can effectively utilize L-arabinose for aerobic growth and also can ferment it to ethanol. Bacteria depend on protein products of the *araBAD* operon, which contains three genes, *araB* (encoding L-ribulokinase), *araA* (encoding L-arabinose isomerase) and *araD* (encoding L-ribulose-5-phosphate 4-epimerase) to convert L-arabinose directly to D-xylulose 5-phosphate through L-ribulose and L-ribulose-5-phosphate [28]). The genes encoding the *E. coli* L-arabinose metabolizing enzymes have been cloned and sequenced [29].

Although some of the yeasts and fungi have been reported to be able to utilize L-arabinose for growth and a few of them can also ferment the pentose sugar to ethanol [30, 31] , the yeast and fungi enzymes involved in the metabolizing L-arabinose to xylitol have not yet been isolated and the genes encoding these enzymes have not been cloned and sequenced. Thus, the logical approach for making our recombinant *Saccharomyces* yeast also able to ferment L-arabinose is to clone and express the bacterial genes in the recombinant yeast. In the following, we briefly describe our results in the cloning and expression of the three *E. coli* genes, *araA, araB, and araD* in *S. cerevisiae.*

Since the three *E. coli* genes will be synthesized by PCR techniques and since it is known that mutations may be more frequently introduced into the DNA synthesized by PCR techniques, in order to insure that the cloned genes are functionally expressed prior to being introduced into the yeast cells we used the following strategies.

First, we obtained three *E. coli* mutant strains and each strain contained a mutation in one of the three genes encoding the synthesis of the three enzymes required for metabolism of L-Arabinose in *E. coli.* As a results, these three mutant strains could only produce white colonies but not red colonies on MacConkey agar plates [32]. If a functional gene had been cloned into each of these mutants and could complement the specific mutation of the mutant strains, the recombinant strains should then produce red colonies on the MacConkey plates.

Second, we selected or made several yeast glycolytical promoters including pyruvate kinase (Pyk) promoter, phosphoglucose isomerase (PGI) promoter, and phosphoglycerol kinase (PGK) promoter that can actively express genes in both *E. coli* and *Saccharomyces* yeasts and also use these promoters to express the *E. coli* genes both in yeast and *E. coli.*

Third, we fused the *E. coli araA* structural gene to the Pyk promoter and resulted in the construction of the recombinant gene Pyk-*araA* . Similarly, we also constructed *PGI-araB* and *PGK-araD.* We also fused each of these recombinant

genes with a yeast 3′ noncoding sequence. According to our early work, the 3′ noncoding sequence of the cloned yeast xylulokinase gene (XK_t) contains effective genetic signals for polyadenylation, mRNA termination, and so on [26, 33]. Thus the intact 3′ noncoding sequence XK_t was fused to the 3′ coding sequences of each of the three recombinant *ara* genes, resulting in the construction of *Pyk-araA-*XK_t, *PGI-araB-*XK_t, and *PGK-araD-*XK_t. Each of these recombinant *ara* genes was cloned into a yeast-*E. coli* . shuttle plasmid and each of the resulting plasmids pYaraA, pYaraB, and pYaraC were used to transform at least two *Saccharomyces* yeast strains. Analysis of the yeast transformants containing each of these plasmids showed that all three *E. coli* genes were expressed and active enzymes encoded by these genes were produced in the yeast transformants during growth and fermentation. Furthermore, back transformation of the *E. coli* L-arabinose mutants by plasmids isolated from the yeast transformants produced *E. coli* transformants that complemented the respective mutations and produced red colonies on MacConkey plates. Analysis of the specific activity showed that the specific activity of L-ribulokinase and L-ribulose-5-phosphate-4-epimerase reached at least 70% of the activity of the wild type *E. coli.*, but that the specific activity of L-arabinose isomerase was only 10% of that of the wild type *E. coli.*

The three recombinant genes, *Pyk-araA-*XK_t, *PGI-araB-*XK_t, and *PGK-araD-*XK_t, were then cloned on a single yeast-*E. coli* plasmid, resulting in the construction of pYaraDBA plasmid. *Saccharomyces* yeasts containing this plasmid still could produce all three active *E. coli* L-arabinose metabolizing enzymes and the plasmids isolated from the yeast transformants could still complement the arabinose mutation of the three *E. coli* L-arabinose mutants. The yeast transformants containing pYaraDBA were then used to ferment L-arabinose and unfortunately very little ethanol was accumulated during the course of fermentation. Currently we are considering to modify the araA gene to make it more actively expressed in yeast. At the same time, we are also trying to clone the L-arabinose metabolizing genes from yeasts and/or fungi to make the *Saccharomyces* yeasts able to ferment L-arabinose.

9
Further Improvement of Glucose-Xylose-Fermenting Saccharomyces Yeasts for Effective, Economical Conversion of Sugars from Cellulosic Biomass to Biofuel Ethanol.

Even though our genetically engineered *Saccharomyces* yeasts are as ideal as can be for cofermenting glucose and xylose, there are at least three types of improvements that could make our engineered *Saccharomyces* yeasts more effective and economical for the conversion of cellulosic biomass sugars to ethanol. One is to make ethanol fermentation faster, particularly xylose fermentation; the second is to reduce the formation of byproducts; and the third is to make the genetically engineered yeasts also able to produce various high-value byproducts.

Some of the ongoing processes to improve our yeasts in these directions are briefly discussed below:

1 Our genetically engineered *Saccharomyces* yeasts rely on the enzymes encoded by the cloned *P. stipitis XR* and *XD* genes to convert xylose to xylulose. However, the cloned *XR* produces a xylose reductase that can use either NADH or NADPH as the cofactor but prefers NADPH. On the contrary, the cloned *XD* gene produces a xylitol dehydrogenase specifically requires only NAD as its cofactor. The general consensus is that if the cofactor requirement for the *XR* enzyme can be modified to using NADH only, the resulting yeast might be able to ferment xylose more effectively by completely regenerating the necessary cofactors within its own xylose fermentation circuit. For testing this hypothesis, we carried out site-specific mutagenesis on the *XR* gene. One mutated gene *XR*(M43) produced a xylose reductase with improved affinity towards NADH cofactor (the ratio of the NADH activity to the NADPH activity is 0.9 for the mutant enzyme while the ratio of the NADH activity to the NADPH activity is 0.63 for the wild-type enzyme). 1400 Yeast transformants containing the plasmid p(LNH33)-M43, which is identical with pLNH33 except that the *XR* gene has been replaced by the *XR*-M43, did ferment xylose slightly faster than the same yeast strain transformed with pLNH33, but not substantially. However, much less xylitol was produced by using the yeast containing the mutant *XR* than the one containing the regular *XR* [34].
2 Recombinant *Saccharomyces* yeast still transports glucose much more preferentially than it transports xylose. Improvement of xylose transport would substantially improve our genetically engineered *Saccharomyces* yeasts in cofermenting glucose and xylose. We have already carried out substantial studies on improving yeast transport of xylose. For example, we have cloned a low affinity glucose transport gene from another yeast species and also some high affinity glucose transport genes from *S. cerevisiae*. The effect of these genes on our engineered yeasts in cofermenting glucose and xylose will be analyzed.

There are still quite a few other improvements that can be made to improve xylose fermentation, including choosing more suitable promoters to express the XYL genes, changing some of the promoters of glycolytic genes which might be heavily regulated by the concentration of glucose present in the medium, etc.

Furthermore, with the successful development of our new and convenient method for integrating multiple copies of genes into the yeast chromosome, the engineered yeasts can be programmed to produce different coproducts in addition to ethanol, and there is no limit on what coproducts can be produced by the engineered yeast. Our goal is to make ethanol produced from cellulosic biomass less expensive than gasoline and we are confident that we can accomplish this goal.

10
Concluding Remarks Particularly on the Importance of Using Safe and Effective Microorganisms for Biofuel Ethanol Production

The selection of a proper microorganism to carry out large-scale production of bulk products is extremely important. Such a microorganism must be absolutely safe and not have any remote possibility of being hazardous to human health or the environment. In addition, for the production of large volumes of low cost products such as ethanol, it is necessary not only to carefully select the microorganism as described above but also to give serious attention to the conditions that are required for culturing the microorganism. It is ideal that the selected microorganism can be effectively cultured under rather extreme conditions so that no other microorganisms, particularly most of the pathogens, can survive under those conditions. This is because the production of such high-volume low-value products cannot afford and also nearly impossible to be totally aseptic and will be unavoidably to release microorganisms to the environment, both the microorganisms used for the production and the contaminates. Currently, three microorganisms have been genetically engineered which are able to ferment both glucose and xylose to ethanol. These are *E. coli* [34], *Zymomonas* [35], and our *Saccharomyces* yeasts. The efficiencies of the best of each of these three engineered microorganisms in fermenting glucose and xylose present in cellulosic biomass are comparable. However, among the three microorganisms, only the *Saccharomyces* yeasts have been used for large-scale production of wine and ethanol for hundreds of years without any adverse effects on environment or human health. Furthermore, only *Saccharomyces* yeasts have been cultured in open vessels for centuries and have not created any ill effects to human health or environment.

In addition to the fact that *Saccharomyces* yeasts have the proven record to be the absolutely safe microorganisms for large-scale production of industrial products, the engineered yeasts can be substantially further improved for more effective fermentation of celllulosic sugars to ethanol, they have a large enough gnome to allow numerous genes to be inserted into its chromosome to produce various by-products, and our successful development of the new reliable method for integrating multiple copies of gene(s) into the yeast chromosome will further ensure the latter possibility. These extraordinary properties will make our genetically engineered glucose-xylose-cofermenting *Saccharomyces* yeasts the overwhelming favorite of the ethanol industry for the conversion of cellulosic sugars to ethanol.

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Research Perspectives for Bioconversion of Scrap Paper

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Avenues for effective research using cellulose as a substrate for bioconversion that can lead to commercialization are discussed.

Keywords. Bioconversion, Paper, Acetate salts, Calcium and magnesium acetate, Anaerobic digestion Bioreactor design, Bioprocessing of paper

1 Introduction

The carbon cycle in nature uses the energy supplied by sunlight for plants to take atmospheric CO_2 to organic fixed carbon that through various chemical or biological steps goes back to atmospheric CO_2. Sugars produced by plants are

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the substrates on which our fermentation industries are based, but cellulose, the most abundant carbohydrate on earth, is much less expensive than sugars. The predominant large-scale bioprocessing of cellulose is the anaerobic digestion of sewage sludges. Such anaerobic processes are slow [1] and the usual product is methane, of little value as pipeline gas because of the cost of removing the carbon dioxide that accompanies it. The profit potential is much greater for other products. For the past two decades, the research community has been on the verge of finding profitable routes for making fuel-grade ethanol from cellulose. The cellulose must provide glucose for the ethanol fermentation. Good yields are impossible with enzymatic hydrolysis unless the cellulose has been pretreated to a small particle size and reduced crystallinity. Acid hydrolysis is technologically simpler but has inferior yields. Although making fuel-grade ethanol must operate on a very large scale to have a measurable impact on national needs for energy, making some other product on a more modest scale could use uncomplicated anaerobic bioconversion technology and benefit economically from the credits for disposal of wastes. Avenues for effective research using cellulose as a substrate for bioconversion that can lead to commercialization should be discussed.

Plant cellulose is always associated with hemicellulose and lignin that protect against microbial attack. Scrap paper is a better substrate for bioconversion because it has already undergone a pulping step that removes most of the hemicellulose and lignin. Other factors are:

1. Scrap paper collected by communities for recycling is inexpensive and may even be supplied with a credit. Fines from the paper industry are waste.
2. Paper fibers can be recycled only 2 to 4 times before they become too short or weak for paper. Those fibers, however, may need no expensive milling prior to bioprocessing.
3. Unlike starch, waste paper has no human food applications.

Some other substrates used for laboratory research not discussed here are shown in Table 1.

At the molecular level, portions of cellulose assume a highly structured crystalline form, while other parts are amorphous. Amorphous cellulose is more easily digested by enzymes than the crystalline parts. The fraction of crystalline cellulose is called the relative crystallinity, an index of digestibility. The crystallinity of commercially available substrates lies between 85 and 90%. Even so-called non-crystalline cellulose has a relative crystallinity of 65%.

Table 1. Substrates used for research

Substrate	Ref.
Mix of ground alfalfa hay and filter paper	[2]
Cellulose-rich municipal solid waste (< 40 mm particle size)	[3]
Bagasse, newspaper and alfalfa hay	[4]
Grass-grain mixture	[5]
Ball-milled filter paper	[6]

2 Possible Products

Many of the products of the fermentation industry could come from the glucose derived from cellulose, but we will focus on direct bioconversion because a separate hydrolysis step or simultaneous saccharification/fermentation, as proposed for fuel ethanol, adds to the complexity and the cost. Of the products from anaerobic digestion of cellulose, acetate itself has commercial potential and is a good model compound. The mixed acetate salts of calcium and magnesium that come from using inexpensive dolomitic lime for neutralization have vast markets for deicing of roads and highways if the cost can approach that of using common salt. The actual cost should consider that the acetate salts are almost benign to the environment while sodium chloride damages vegetation, can ruin water supplies, corrodes automobiles and shortens the lifetimes of bridges, underground cables and the highways to which it is applied.

3 Laboratory Reactor Design

Research with anaerobic cultures can be in batch or continuous culture. Fed-batch anaerobic reactors are not known to us but may very well be feasible. A reliable technique for batch cultures uses serum bottles sealed with butyl rubber stoppers and crimp sealed with an aluminum cap. Anaerobic microorganisms in batch flasks are mainly cultured by Hungate's methods [7] that are widely accepted in the research community. We will not describe these techniques but will focus on the different continuous culture apparatuses that have been developed over the years.

Maintenance of an anaerobic culture of microorganisms is extremely difficult in continuously operated laboratory bioreactors. Designing and operating a continuous anaerobic bioreactor are not easy. Subtle differences in process configuration such as phasing, staging, biofilms, granules, gas-phase management and combinations thereof can profoundly impact anaerobic process performance [8]. The main problems are:

1. Protection of the feed stream from oxygen.
2. Reduction of the medium to a redox potential of at most –50 mV.
3. Removal of a slurry from the reactor without clogging the tubing.

Pumping is slow because of the long liquid residence time and insoluble cellulose tends to settle out and clog the tubing.

4. Replacement of liquid and gas removed from the reactor with anaerobic gas. Oxygen is otherwise sucked into the reactor and poisons the culture.
5. Maintenance of a uniform reactor temperature. Anaerobic reactors are often operated at 37 to 39 °C, or higher. With just a single heating tape around the reactor, the cold parts of the unheated reactor walls may slow the growth of the microorganisms (personal experience). Reactor vessels should be insulated.
6. Complicated pH relationships of a carbonate-buffered system because production of organic acids lowers the pH while CO_2 may be consumed by the

microorganisms thereby raising the pH (operation of a pH-auxostat is highly challenging).
7. Development of a reliable stirrer system. Heavier cellulose particles settle and a too-thick cellulose suspension squanders mixing power.
8. Oxygen-free and sterile sampling technique for the reactor broth.

The reactor must have means for withdrawal of liquid samples with a representative cellulose concentration.

9. Inoculation should be aseptic with no exposure of the inoculant to oxygen at any time.
10. Additives must be free of oxygen and sterile.

The zero tolerance for oxygen of strictly anaerobic microorganisms is a major issue. The reason for the intolerance to oxygen is the deficiency of the microorganisms in superoxide dismutase and catalase [9]. Reduced flavoproteins and reduced sulfur-iron proteins that contact oxygen form hydrogen peroxide and superoxide radicals that are toxic. In aerobic microorganisms, these compounds are quickly decomposed by catalases and superoxide dismutases. While facultative anaerobic microorganisms only lack catalase activity, strict anaerobes lack both enzymes and will die if exposed to oxygen.

Hoover et al. [10] set the stage for a whole series of reactors by designing an *artificial rumen reactor* later adapted by other investigators. This anaerobic reactor allowed withdrawal of both a clear liquid stream and a suspension resulting in different residence times for solids and liquid. The reactor was later used by other groups with almost no changes [2–5]. There are usually multiple fairly small reactors allowing several experiments in parallel. One system consisted of eight magnetically stirred glass reactors each with a working volume of 530 ml [4]. An earlier artificial rumen reactor was operated at conditions encountered in the rumen of a cow [11]. A similar reactor consisted of five reactor vessels of 400-ml working volume and was not equipped with a filter unit [12, 13]. Both systems had a gas collection tube. Another system consisted of one 1.5-l reactor vessel [5]. The rest of the design was adapted from Hoover et al. [10]. A departure from earlier systems was a reactor with a working volume of 940 ml and with a rather unique and cleverly designed feed system that will be discussed later [14].

Controlled experimentation is not easy because several bioreactors in parallel are not likely to reach identical steady states. This means that there is no common starting point when making changes. One way to circumvent this problem is to have one large bioreactor that has its effluent split to feed a number of other bioreactors. A mother-daughter reactor system has been used to provide a constant feed so that the daughter reactor can start at a controlled condition while responses to substrate perturbations are explored [15]. Another reactor configuration is the upflow sludge bed reactors (e.g. [16]), but this is impractical for thick suspensions of cellulose.

There is a clear trend to small reactors. These provide little room for the sensors needed for modern monitoring and control and often cannot be sampled without depleting the contents; a better working volume would be in the range

of 8 l to accommodate the various sensors and other devices for liquid withdrawal, gas recycling, settling tubes, and the like. At typical dilution rates, a larger size allows for more accurate pumping.

3.1 Head Gases

The necessity to keep the reactor anaerobic leads to the use of sparging gases. A good choice is a mixture of so-called anaerobic gas consisting of 5% H_2, 10% CO_2, and balanced with N_2. This is rather expensive, and pure CO_2 is often used instead. Nitrogen and argon have also been used, but we spent one and a half years with almost no success trying to grow anaerobic microorganisms using nitrogen or argon gas. We blame this on the stripping of CO_2 that is essential for growth of rumen bacteria. We later kept the head space of our reactor at a slight positive pressure of CO_2 and also sparged the broth when a challenge by oxygen was suspected. Gas can be managed inside the bioreactor with a football bladder filled with anaerobic gas, and connected to the feed and reactor vessel [12, 13]. This allows an exchange of gas without influencing the carbon balance because of the continuous addition of CO_2 gas to the reactor (CO_2 is a substrate for acetogens).

A heated glass column filled with copper filings was introduced in the early days of anaerobic research. The idea is to remove residual oxygen from the gas stream as it oxidizes the copper filings. The quality of industrial grade CO_2 is very high, and in our opinion does not require additional reduction.

3.2 Feeding Methods for Cellulose

Feeding insoluble cellulose into large vessels is easy, but it is a difficult task for small laboratory reactors. Cellulose has been fed manually; we were among the first to feed dry cellulose under computer control directly into the reactor. Most systems reported so far have fed a cellulose slurry into the reactor. High concentrations of cellulose suspended in the slurry tend to settle in the reservoir and narrow parts of the tubing causing clogging. This is especially bothersome since anaerobic reactors operate at a much longer retention time than their aerobic counterparts, making it more difficult to reach a steady state after a disturbance. Because of the long residence time, anaerobic laboratory reactors often run unsupervised for long periods of time. The first artificial rumen reactor was supplied with a dry feed of cellulose fed manually into the reactor [11]. This method is still in common use if the research plan calls for the feed of a more granular substrate such as ball-milled hay or paper [4, 5].

Feed by injection from a continuous recycle loop operated with a large well-mixed feed reservoir connected to one branch of a tee-fitting [12, 13]. Another branch of the tee led to the bioreactor. The tee itself was in a loop of tubing with a peristaltic pump to circulate fresh feed through the loop and back into the feed reservoir. Solenoid valves were installed downstream of the tee-fitting and in the branch leading to the reactor. The valve leading to the reactor was closed

whenever the valve in the recycle loop was open and vice versa. This allowed pulse-wise injection of liquid into the reactor, while the high recycling rate avoided settling of the insoluble cellulose in the tubing. The feed system was used for a slurry of 1% (10 g/l) microcrystalline cellulose, Avicel PH-105, to the reactor.

Gravity feed through a nitrogen-activated tube valve used a well-stirred feed vessel placed above the reactor [17]. A section of compressible tubing in the feed line connected the feed reservoir to the reactor. The compressible tubing was placed inside a rigid container that could be pressurized with nitrogen gas. The tubing contracted when squeezed by nitrogen, stopping the flow of the slurry. Figure 1, a sketch of the valve setup, shows the valve in the horizontal position. The feed liquid consisted of 0.1% (1 g/l) ball-milled cellulose and the feed reservoir had a volume of 10 l.

Feed with a gas/liquid segmentation system interrupted the feed stream with small pockets of inert gas to avoid the settling of cellulose in an entire section of the tube [18]. There was never enough cellulose in a small section to cause clogging. Choosing the feed tube diameter appropriately prevented the inert gas segments from migrating and combining. A schematic of the feed system is shown in Fig. 2. A peristaltic pump with a small tube delivered inert gas, and a second pump entrapped small gas pockets in the liquid stream.

A similar system was already commercially available for a different application. A progressive cavity pump for feeding was placed in the bottom outlet of the feed reservoir [19]. It was claimed that the feeder delivered slurries of 0.5 to 6% (5–60 g/l) cellulose. This is remarkable because we were never able to suspend more than 30 g/l of cellulose in a vessel; no data were presented to support the claim.

The cellulose concentration in the bioreactor cannot be greater than the concentration in the feed slurry unless there is provision to decant the broth to

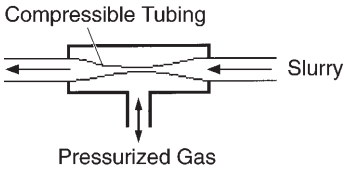

Fig. 1. Gas activated feed valve for cellulose slurries [6]

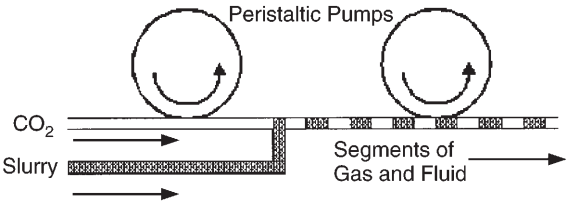

Fig. 2. Schematic of gas-liquid segmentation system [14]

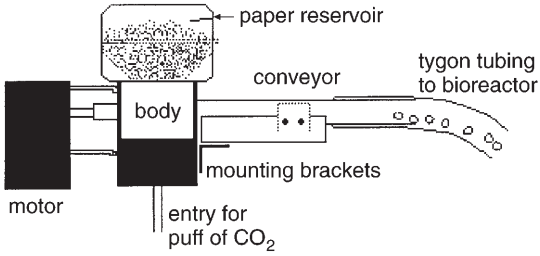

Fig. 3. Feeder for fragments of cellulose [20]

remove excess water. We circumvented the slurry problem by inventing small feeders with rotating barrels to move larger fragments or with a brush to move powders of solid cellulose [20]. The feeder for the chaff from a paper punch is shown in Fig. 3. Both the reservoir and the barrel rotate so that the paper flows smoothly. Our entire system was placed in a plastic glove bag, and a puff of CO_2 when the feeder was turned on displaced stale gas from the feeding system.

3.3
Systems for Removal of Fermentation Broth

A vexing problem is the removal of the cellulose slurry from the reactor. With vigorous stirring, the overflow stream is assumed to have the same concentration as the fermentation broth. An overflow can allow spent ball-milled filter paper to run into the waste bottle [17]. A filter unit has been used to retain solids and to provide clear liquid from the reactor; suspended cellulose was drained through an overflow tube [2–4]. The reactors were adapted from the design of Hoover et al. [10]. One filter was a cylinder (6 cm long, 1.5 cm diameter) of 40-mesh stainless steel wire cloth with rubber stoppers at each end [4]. The cylinder was wrapped in three layers of 30-mesh nylon monofilament cloth held in place by the rubber stoppers. The inside of the filter was filled with glass fiber and a glass tube penetrated one rubber stopper to collect the filtered liquid.

We found that an inclined overflow tube would allow most of the cellulose to settle back so that clarified liquid could be passed through an analytical system. When the dilution rate was too slow to provide sufficient sample, the sample stream plus small amounts of salt (formed from neutralization in the analytical system) could be recycled to the bioreactor. Cellulose was withdrawn separately with a smaller tube that allowed no settling. Independent removal of clarified liquid and cellulose slurry could be controlled to hold a setpoint for cellulose concentration, but we have not used this other than to carry out some manual adjustments.

3.4
Measurement of Oxidation/Reduction Potential

While the pH of a solution is a measure of the hydrogen-ion activity, the redox potential is a measure of the electron activity. Although it is well accepted that

rumen bacteria need a low redox potential, the reason why the redox potential has to be lower than –50 mV is not known. Methane bacteria are among the least tolerant organisms to higher redox potential and generally do not grow above –300 mV. We installed a redox potential electrode in our reactor and quickly discovered that a culture under stress (low cellulose concentration, high dilution rate, etc.) was not able to keep the redox potential low. The redox reading is an early indicator of the well being of the microbial population. Recordings of the redox potential show whether the culture has been accidentally poisoned with oxygen when the operator is not present. Measurement of redox potential is one of the most important aids to research on anaerobic processes because a rise indicates immediately that something is wrong or that a variation in techniques is unacceptable.

3.5
Other Instrumentation and Control

The level of sophistication for instrumentation of an anaerobic process has been advanced considerably with a mass spectrometer membrane probe for the measurement of hydrogen, methane, oxygen and carbon dioxide [21]. Interfacing a bioreactor to a computer is becoming quite routine. Sophisticated control algorithms are rare for anaerobic processes, and the interplay of the various signals with regard to the biochemistry of the process is interesting. After discovering that pH was unsuitable for auxostatic operation at neutral pH because of the minute effect of concentration of organic acids so far from their p*K*s, we searched for another measure of product concentration. Although the usual chromatographic determinations of organic acids are precise and accurate, continuous monitoring using automation is expensive and cumbersome. We fabricated a device for continuous estimation of the concentration of the organic acids in the effluent stream [22]. The basic concept is to control the pH in the bioreactor and to pass a sample stream through a two-head pump to add mineral acid in exact proportions. Selection of an amount of acid that brings the pH near the p*K* of acetic acid allows that pH to reflect the concentration of organic acids.

A torsion dynamometer that measures torque on the mixing shaft would be highly desirable. This would relate to viscosity that depends to a major extent on the concentration of solid cellulose. Auxostatic control of viscosity would be a novel way to operate the bioconversion of cellulose with high promise for selecting organisms that break down cellulose rapidly.

3.6
Dilution Rate

Dilution rate is a major factor in design, operation and profitablity of a continuous bioreactor. When the main nutrient is a solid, dilution rate and detention time of the solids are two separate but interrelated issues. Hydrolysis of the cellulose continues as long as it is in the bioreactor, but the rates should change as the amorphous regions react more quickly leaving a higher proportion of the

more crystalline regions. It can be harmful to accumulate cellulose that reacts relatively slowly because it adds to the viscosity of the fluid impairing mixing and consuming more mixing power. All systems of this sort provide for some bleed of solids to avoid build up of inert materials. This bleed percentage depends on the fraction of inerts entering the bioreactor, and a small safety factor should insure that highly crystalline cellulose leaves the system. The setting of this bleed rate for solids should benefit from computer modeling after the hydrolysis rate constants have been defined more accurately.

Concentration of solid cellulose should be as high as practical in terms of mixing for an industrial process. Mixing need not be vigorous for an anaerobic process, but the power required will rise sharply as the medium becomes too high in solids concentration. This should be easy to monitor and control for the industrial process. A wattmeter in the electrical circuit of the mixer would indicate when too much power was being consumed, and the feeding of fresh cellulose could be halted or decreased to let hydrolysis reduce the viscosity. A laboratory bioreactor has different restraints. It would be wise to operate at a somewhat lower solids concentration to attain more homogeneity and to encounter fewer problems with pumping and sampling.

The solutions that enter and leave the bioreactor are closely matched but need not be exactly equal because of evaporation and biochemical reactions that evolve gases or produce water. Dilution rate is the entering volumetric rate divided by the volume of the reactor contents and has the units of reciprocal time. In this case, it is convenient to consider the liquid and solids separately. The feed solution must provide balanced nutrition – everything except the cellulose which is the carbon energy source. These other nutrients should be in excess because the growth rates of the various organisms can change. Too much excess can impair the recovery of the product, but the spent broth after product recovery can certainly be recycled to some degree.

Population shifts are expected in mixed culture processes, and continuous culturing promotes selection of organisms that adapt well to the conditions. Guessing at a suitable dilution rate is unlikely to hit the mark, and the target must move as the organisms change or as there are variations in the substrate as would be likely with a plant handling waste paper. Instead of a system for which the operator decides on the dilution rate, it would be much better to match feeding to the microbial activity. This method of continuous culturing is termed an *auxostat*. Some index of microbial activity must be monitored, and the feed rate adjusted to fix its value.

Some possible types of auxostats for continuous conversion of paper to organic acids are:

1. Feed a slurry of cellulose plus nutrients and vary feed rate to hold viscosity constant. This couples solid with the solution but might be suitable for commercialization.
2. Feed cellulose at a rate that keeps its concentration roughly constant and handle the other components separately with a pH auxostat. The solution feed rate would determine pH by buffering and dilution of the organic acids that are formed.

3. Same as the pH auxostat but substitute a setpoint for concentration of organic acids and dilute back with the feed stream.

Auxostatic operation of a continuous reactor system holds the promise that the culture can be operated safely at a high specific growth rate [23]. A key state variable such as the concentration of the desired fermentation product is fed back to the controller of the feed pump and the feed rate is adjusted to maintain the setpoint of product within the reactor. When a culture tends to increase the concentration of product, the controller causes the pump to speed up, washing parts of the culture out of the reactor. If the microbial population is unable to maintain the desired concentration, the pump is automatically slowed down while the organisms excrete product. Once a steady state is established, the specific growth rate of the organisms equals the system dilution rate [24]. This operating mode has the advantage of washing out the slower growing microorganisms from the reactor leaving more substrate for those that grow faster. It is also obvious that a faster growing, contaminating population that also accumulates the factor being controlled will challenge the established culture to cause its wash-out from the system. Auto selection of yet faster growing cultures is the logical result.

Supplying cellulose as the only carbon and energy source to the reactor as well as simultaneous feedback of the acetic acid concentration to the controller assures that auto selection occurs in the direction of faster cellulose degrading organisms. This scheme also carries the advantage that shock loads, such as poisonous ink associated with paper, do not result in the loss of the entire microbial population by wash-out, a phenomenon feared by all operators of chemostats [25]. Moreover, the auxostat scheme may select a culture that overcomes poisoning or other threats. Since new organisms or variants are welcomed to challenge the existing population in their attack on waste paper, expensive sterilization and aseptic operations are more of a disadvantage than a benefit to the system. A deeper insight into the auxostat principle can be found in various reviews [23, 26].

An anaerobic reactor has been operated as a pH-auxostat [17], but we question whether this can lead to a commercial system. The pH of a reactor broth decreases if CO_2 is bubbled through the broth. The sparging gas was 5% H_2, 10% CO_2 and 85% nitrogen. When CO_2 is fixed by the organisms, the pH is expected to rise, causing the auxostat to stop feeding. The culture is therefore not rewarded by production of acetate (remember that most of the acetate will remain in its deprotonated form at pH close to neutrality). We had some disappointing experiences with a pH-auxostat during the early stages of our research.

We have operated an auxostat by monitoring organic acid concentration and controlling by dilution with fresh feed. Feed is slowed when below the setpoint to allow the organisms to produce more acids to restore their concentration. A rise in concentration causes the feed pump to speed up to dilute the acids. This was the status of our research when the project was put on hold while a search was initiated to find a new research assistant who could handle the engineering, biochemistry and microbiology of this complex system.

4
Microbial Culture

It might be possible to find a suitable culture for bioconversion of paper in nature or in an environment such as an anaerobic digestor in a waste treatment plant. Fully satisfactory cultures are available for mimicking existing bioprocesses, but the conditions in a process for producing a commercial product such as acetate are sufficiently different that finding a culture that grows rapidly enough while producing that product is a serious challenge. We tried organisms from soil, from bottom sediments of lakes, from waste treatment plants, and from rumen fluid obtained from a local slaughter house. The rumen fluid was better than the other samples, but the rates were much too slow for commercialization. Two pure cultures have been combined for improved anaerobic digestion [27]. Far better than any other culture that we tried was a tri-culture provided by Drs. Wolin and Miller of the Wadsworth Laboratories of the New York State Department of Health [28]. A key feature was an organism with the specific role of scavenging any traces of oxygen to hold the oxidation-reduction potential low enough for the other organisms to thrive.

Halotolerant organisms will be needed as the setpoint of acetate is raised. Our reactor operates at neutral pH with a system for tight control. Using lime to neutralize the organic acids would result in the desired salts for deicing of highways, and a product of suitable quality could come from evaporation of the liquid. We hope that gradual increases in the setpoint for acetate concentration will select organisms that can tolerate the elevated concentrations of salts.

5
Other Nutrients

The source of nitrogen must be evaluated carefully in terms of cost. Inorganic compounds such as ammonium salts or nitrates of the grades used as fertilizers are not expensive but may be inhibitory [29]. However, complex nutrients such as corn steep liquor or distillers solubles often supply some ill-defined but highly advantageous growth factors. Municipal sewage sludge is seldom used as a nitrogen source for commercial bioprocesses but deserves serious consideration if the product is a crude grade of acetate salts for deicing of roads.

We advise keeping the concentration of sulfate low in a laboratory bioreactor. We used sulfate salts in the medium and used sulfuric acid to adjust pH in the sample analytical system with some recycle, and the stench was overpowering. The high concentration of sulfide also encouraged precipitation that gave the broth a black color. Furthermore, evolution of massive amounts of hydrogen sulfide from a commercial system would entail costly measures for protection of the health of the plant operators.

6
Models

We enthusiastically endorse models as an aid to this type of research. Our model of acid-base equilibrium was essential for the design and operation of

our device for monitoring the concentration of organic acids. There are a number of models of anaerobic digestion that will not be reviewed here. There are several recent articles that are good starting points for exploring such models [30–33].

7 Downstream Processing

A main obstacle to recovery of organic acids is low product concentration. While a high production rate is important, a high product concentration is even more important for cost-effective purification of the acids. A multi-stage counter-current system at low pH for extracting the organic acids into a tri-*n*-octylphosphine oxide (TOPO)/kerosene phase has been demonstrated and analyzed [34]. The kerosene solution is less expensive and easier to handle than TOPO alone which was identified as the best agent. A concentration of 10% was favored since the viscosity was the lowest at that point, and pumping would be easier. Distillation of the TOPO/kerosene phase is a way to recover the desired organic acids. Direct distillation of the fermentation broth with no extraction step is not practical. The biomass and residual cellulose could clog the lines, and the heat of vaporization of so much water from the dilute solution makes energy costs too high.

A reverse osmosis unit has been proposed for the recovery of organic acids [4]. The plant under consideration was assumed to have a cellulose feed rate of 44,000 kg (dry) of municipal solid waste per hour. The flow to the unit consisted of 3% short-chain organic acids (usually referred to as VFA for *volatile fatty acids*). It was also estimated that the unit would concentrate 36,500 kg of VFA to a 25% solution. The total estimated capital cost for the reverse osmosis unit was 26.5 million dollars, assuming a membrane life time of 2 years. This estimated price was $ 0.378 per kg of concentrate leaving the system.

Recovery of propionic and acetic acid from a fermentation broth by electrodialysis was investigated with a dialysis unit fed with a mixture of acetic and propionic acid and varying background salt concentrations [35]. Electrodialysis was best at neutral pH, good news for a typical anaerobic process. A solution of 1.5% (w/v; 0.156 M) sodium propionate at pH 7.0 was enriched to a final concentration of 13.8% (w/v; 1.436 M). The final concentration dropped with decreasing pH, reaching 12% (w/v; 1.249 M) at pH 6.0. The paper reports that the final concentrations of product on the concentrate side of the unit were reduced, but acid recovery improved when background salts such as NaOH were present. This is caused by a decrease in low-current shut-off. This finding has a good and a bad side. Improved recovery would allow reduction of the product concentration in the reactor to a minimum during extractive fermentation, minimizing the effects of product inhibition and, therefore, raising reaction rates. On the other hand, a decrease in final concentration of the enriched product would cause a cost increase of downstream processing. Whether or not the use of an in situ electrodialysis unit is of advantage will depend on the flow chart of the actual process. It should be noted that the auxostat principle is compromised if product is removed directly from the reactor. The auxostat control

algorithm could be modified to compensate for removal of the factor being controlled, but this has not been reported yet.

8 Proposed Processes

We envisage that a commercial system would have large concrete reactors with steel covers. Agitation would be barely sufficient to provide for a mixed liquid phase. Any heavier paper particles would settle into a hopper that would intermittently be subjected to brief intense shear blending. A small bleed would ensure that inert materials would not build up. Solids concentration should be high to insure maximum productivity with reasonable reactor size. It is not essential to control the solids concentration exactly; there could be constant feeding of solids with adjustments to the rate during each shift to account for changes in bioreactor slurry concentration. The product would be in relatively clear liquid withdrawn near the surface through an inclined settler.

Because of advanced computer control (auxostat mode) and an anaerobic culture, sterilization of the feed, aseptic techniques, and sparging of the broth are not required. Using standard equipment mills, waste paper would be shredded directly into the reactor. This avoids expensive pre-suspending of the paper in a separate tank, prevents microbial contamination of the feed stock, and circumvents difficult pumping of paper slurries. Once the acetic acid has been extracted, some of the broth could be recycled back to the reactor. If the product were mixed calcium/magnesium salts, the solution could be concentrated to form crystals and sold. While the process control is sophisticated, the overall system represents a low cost approach for the production of acetic acid mixed with small amounts of other acids. The obvious application is acetate salts for deicing of highways.

Whether this reactor could be operated using a specially formulated culture of defined organisms or a mixed microbial population and how the reactor could be controlled are unanswered questions. Because of the low tolerance of some anaerobic microorganisms towards cations such as Na^+, NH_4^+ and K^+, we envisage the use of dolomitic lime or calcium hydroxide for control of the reactor pH. If the reactor is an auxostat based on product concentration, organisms that thrive at high concentrations of acetate salts should be selected automatically.

None of the options for product recovery are without problems. Electrodialysis to reach a concentration suitable for deicing of highways would be less expensive than a method for producing dry material. However, the solution would have to be dispensed from tank trucks instead of common trucks. Solar evaporation during the summer months is worth considering because of its low cost (except for purchase of flat land).

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Ethanol Production from Renewable Resources

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Vast amounts of renewable biomass are available for conversion to liquid fuel, ethanol. In order to convert biomass to ethanol, the efficient utilization of both cellulose-derived and hemicellulose-derived carbohydrates is essential. Six-carbon sugars are readily utilized for this purpose. Pentoses, on the other hand, are more difficult to convert. Several metabolic factors limit the efficient utilization of pentoses (xylose and arabinose). Recent developments in the improvement of microbial cultures provide the versatility of conversion of both hexoses and pentoses to ethanol more efficiently. In addition, novel bioprocess technologies offer a promising prospective for the efficient conversion of biomass and recovery of ethanol.

Keywords. Cellulose, Hemicellulose, Lignocellulose, Yeast, Pretreatment, Simultaneous saccharification and fermentation (SSF), Ethanol, Xylose, Tower fermentor, Air-lift loop fermentor

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1 Introduction

Over 1.3 billion gallons of ethanol are produced annually in the US, primarily from corn. For the production of alcohol from corn, both wet- and dry-milling processes are used with wet-milling accounting for about 60% of the total ethanol production. For dry-milling, grains are ground to reduce particle size prior to conversion to ethanol. Typically, starch is gelatinized with steam, liquefied to dextrin with amylolytic enzymes, saccharified with glucoamylase, and fermented with yeast to ethanol. Ethanol is distilled from the beer and the resulting stillage is dried or fed wet to animals. Where corn is not readily available, wheat, barley and other starchy materials can be used as feedstock for ethanol production. In addition to starchy substrates, other feedstocks, such as beet molasses, cane molasses, and sugar cane juice, can be used as substrates for industrial ethanol production.

Another potential feedstock for ethanol production is the "lignocellulosic biomass". Lignocellulosic biomass is the most plentiful of all naturally occurring organic compounds. It includes such materials as wood, herbaceous crops, agricultural and forestry residues, waste paper and paper products, pulp and paper mill waste, and municipal solid waste. Unlike starchy materials, lignocellulosic biomass is structurally complex. The conversion of this material into ethanol has been the subject of intense study over the last 20 years.

Native lignocellulosic materials are heterogeneous, containing cellulose, hemicellulose, lignin, ash, protein, and a wide array of minor extractives. Cellulose is a linear high molecular weight polysaccharide composed of two residues (β-1,4-linked glucose, known as cellobiose) in repeated units. Complete hydrolysis of cellulose yields the sugar, glucose. On the other hand, hemicellulose has a much lower molecular weight than cellulose. It is a heteropolysaccharide that is composed of various hexoses (e.g. glucose, mannose and galactose), pentoses (D-xylose and L-arabinose), uronic acids, acetic acid, and other minor sugars. By definition, hemicelluloses are short branched chain heteropolysaccharides of mixed hexosans and pentosans that are much easier to convert to their sugar constituents than cellulose.

2 Fermentation of Hexoses

For the efficient conversion of starchy materials to ethanol, productivity is the major concern. Since the primary sugar from starch is glucose, the biocatalyst used is almost exclusively the yeast, *Saccharomyces cerevisiae*. *S. cerevisiae* and related species have the ability to utilize a wide range of hexoses such as glucose, fructose, sucrose, galactose, maltose, and maltotriose to produce a high yield of ethanol. In addition, closely related species such as *S. uvarum* (*S. carlsbergensis*) and *S. distaticus* are able to utilize melibiose and starch, respectively. Another microorganism, *Zymomonas mobilis*, has been considered as an alternative biocatalyst. This bacterium has advantages over *S. cerevisiae* due to its ability to employ the Entner-Doudoroff pathway anaerobically that converts pyruvate to ethanol and CO_2 as the sole means of energy generation (see [1] for review). This pathway yields only one mole of ATP per mole of sugar consumed. In order to compensate for this low energy yield, glucose is metabolized at a very high rate resulting therefore in high ethanol productivity [2, 3].

2.1 Process Improvement

In order to increase productivity, thereby reducing the cost of ethanol fermentation, various alternatives to traditional fermentation techniques, including biocatalyst improvement, have been studied.

2.1.1 *Strain Improvement*

Various techniques are employed to obtain the improved biocatalysts for potential improvement. These include the following: mutation and selection, hybridization, protoplast fusion, and recombinant DNA methods. One example of strain improvement is the development of an ethanol-tolerant yeast strain, *Saccharomyces* 1400, through protoplast fusion of *S. distaticus* and *S. uvarum* as reported by D'Amore et al. [4]. This yeast strain was used as the biocatalyst by Krishnan et al. [5] for the rapid fermentation of high concentrations of glucose

to ethanol. It was also used as the host in the creation of a recombinant xylose-fermentation yeast strain by Ho and Tsao [6]. Another example of strain improvement is the isolation of the high flocculent yeast strain, *S. uvarum* U4, by Gong and Chen [7]. This high-ethanol and high-osmotic pressure-tolerant flocculent yeast has been used in a packed yeast column for continuous ethanol fermentation [8–12].

2.1.2
Continuous Ethanol Fermentation

One effective way to increase ethanol productivity is to employ a continuous process using an immobilized biocatalyst. Living cells can be entrapped within porous solid supports, such as calcium alginate, carrageenan or polyacrylamide. Surface adsorption on solid support is another method of cell immobilization that has been widely employed. In surface adsorption, microbial cells are adsorbed onto the surface of wood chips, bricks, synthetic polymers, or other materials that have a large surface area (see [13] for references). Compared to a conventional batch free-cell system, the immobilized cell system has the advantage of being able to maintain a much higher cell density in the fermentor thereby increasing ethanol productivity. Continuous ethanol fermentation can also be carried out in a tower fermentor packed with flocculent yeast cells.

2.1.2.1
Tower Fermentor

Tower fermentors were developed for the beverage industry in the 1960s to provide a continuous fermentation system that would be more productive and efficient than the batch system. Their special feature is the use of a flocculent yeast strain in a narrow vertical tubular reactor. Medium containing both nutrients and substrate flows into the bottom of the fermentor and is fermented as it migrates up through a dense bed of yeast cells. The fermented medium passes through a settler before exiting. The purpose of the settler is to provide a quiescent zone where yeast flocs, buoyed up by carbon dioxide evolution, can settle back into the reactor. Figure 1 shows the configuration of such a fermentor with packed yeast cell aggregates.

For high flocculent yeast strains such as *S. uvarum* U4, a near solid yeast plug is formed at the lower region of the tower fermentor and above this region lies a mixed zone containing smaller yeast flocs and newly formed yeast particles (Fig. 2). The bed height of the yeast plug, the size of yeast flocs, and the concentration of flocs in the mixed region are the functions of flow rate, CO_2 evolution, and substrate density [10]. During fermentation, reactor operation can approach plug flow behavior through the yeast bed, analogous to a fixed-bed reactor. At low flow rates and low CO_2 evolution, the mixed section above the plug may be nearly floc-free. On the other hand, very high flow rates and high CO_2 production will cause the bed to disintegrate. The reactor then becomes a mixed-flow reactor, analogous to a fluidized-bed reactor [12].

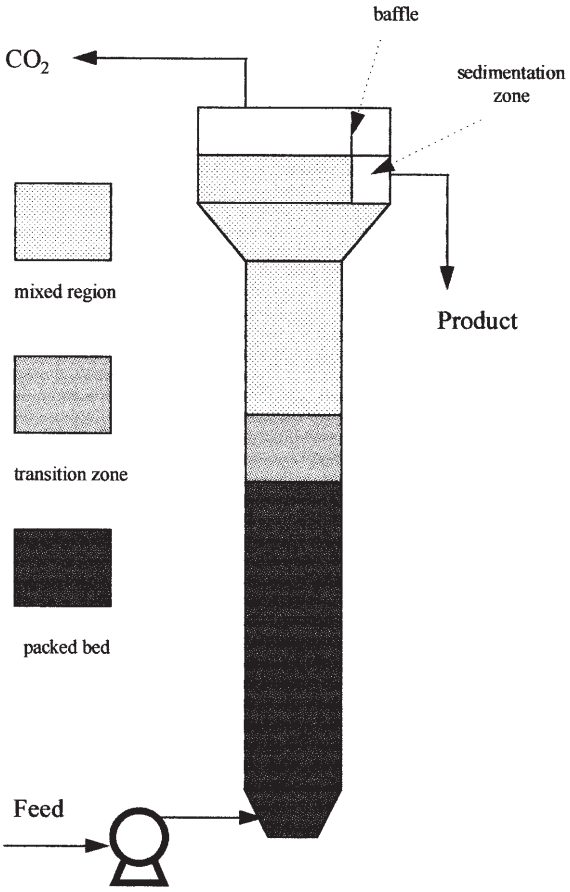

Fig. 1. Schematic configuration of a tower fermentor with *S. uvarum* [12]

Continuous ethanol fermentation using a tower fermentor with high flocculent yeast strains has been studied in depth (see [13] for pertinent references). Overall, the system has the following characteristics:

(1) A much higher cell concentration (70–100 g dry cell/l) than the immobilized cell system (30–50 g dry cell/l).
(2) Self-maintenance, without need for immobilization.
(3) Highly resistant to contamination due to the very high yeast cell density and the rapid rise in ethanol concentration.
(4) Long-term stability due to the self replenishing of fresh yeast cells.
(5) High ethanol productivity.
(6) No requirement for centrifugation and agitation equipment and, therefore, reduction in labor and maintenance costs.
(7) Lower capital expense due to the higher throughput.

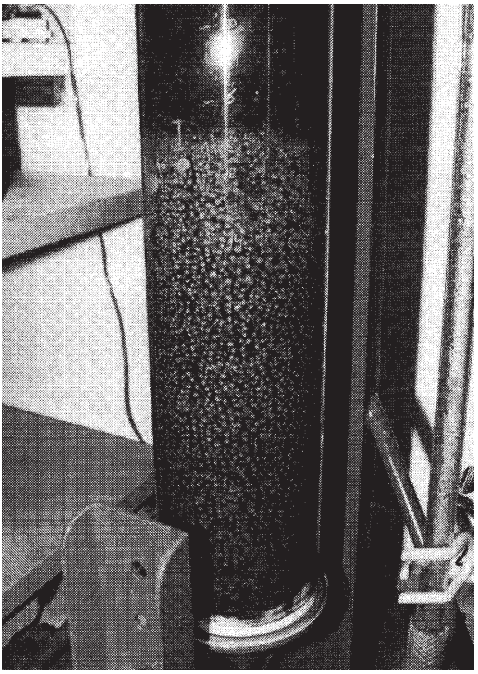

Fig. 2. Heavy yeast flocs in a tower fermentor [9]

2.1.3
Simultaneous Ethanol Fermentation and Recovery

Ethanol inhibition is one of the principal factors restricting fermentation rate, yeast activity, and the maximum ethanol concentration achievable during ethanol fermentation. In order to maintain fermentation efficiency, relatively dilute substrate concentrations of 200 g/l or less are often used resulting in a final ethanol concentration of around 100 g/l after 48 to 72 h. Therefore, high ethanol tolerance is a desirable characteristic of yeast strains in order to improve the overall process economy [4]. Another approach to minimize ethanol inhibition is to remove ethanol from the fermentation broth during fermentation.

There are many different approaches for in situ ethanol removal during fermentation. These approaches include vacuum distillation, solvent extraction, membrane reactors, and gas stripping (see [14] for review). Gas stripping of ethanol during fermentation offers advantages in terms of its effectiveness and ease of operation [15]. Ethanol can be recovered from the carrier gas stream by adsorbing onto activated carbon [16] or by condensation of the recycled gas stream under low temperatures [17].

In order to study in situ ethanol production and recovery, Tsao et al. [18] conducted ethanol fermentation in an air-lift loop fermentor with a side arm (ALSA) using CO_2 to strip ethanol from the fermentor. Analogous to the traditional air-lift fermentor, the ALSA consists of an inner tube that enhances the mixing and mass transfer characteristics (see Fig. 3 for fermentor configuration

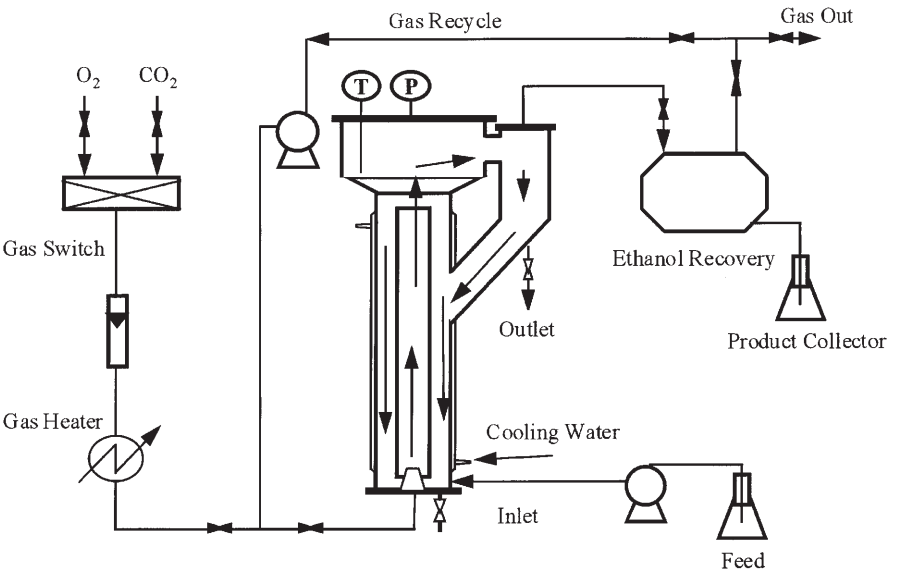

Fig. 3. Schematic configuration of an air-lift loop fermentor with a side arm (ALSA) integrated with CO_2 stripping of ethanol [18]

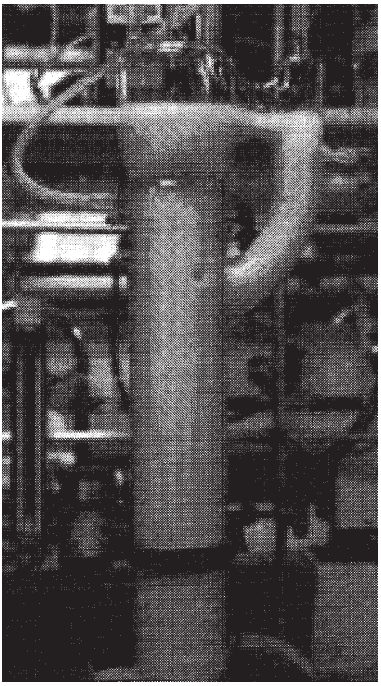

Fig. 4. Laboratory-scale ALSA fermentor [18]

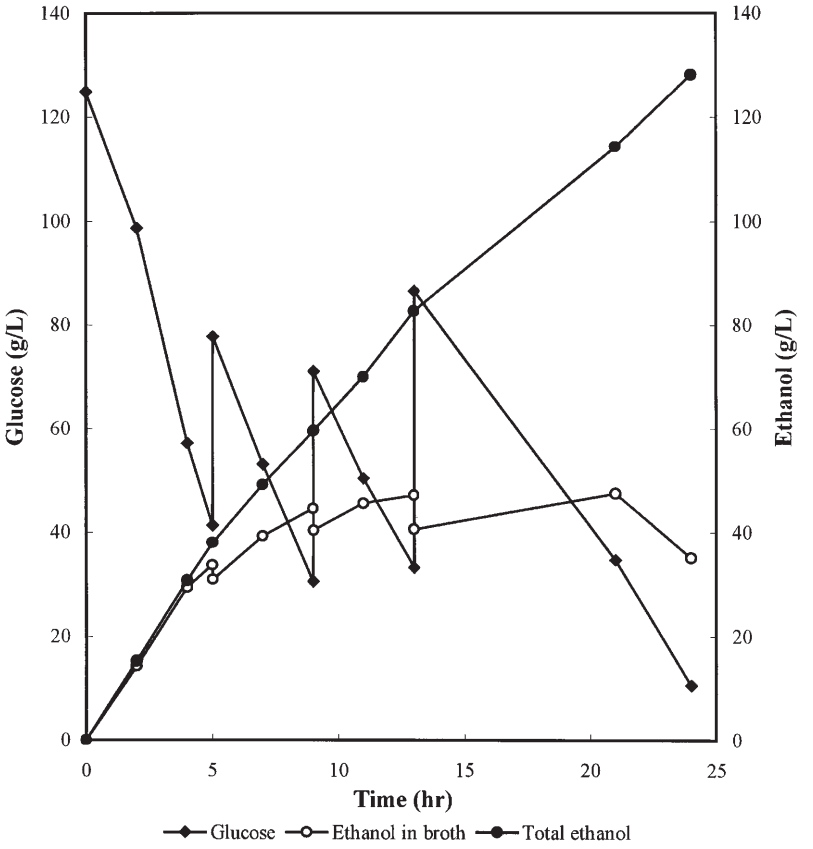

Fig. 5. Fed-batch glucose fermentation with *S. uvarum* in an ALSA fermentor integrated with CO_2 stripping of ethanol [18]

and Fig. 4 for a laboratory-scale fermentor). The side arm serves as an external loop that improves liquid circulation and mass transfer. With this reactor to carry out fermentation and gas stripping of ethanol, *S. uvarum* U4 is able to produce over 130 g/l ethanol within 24 h (Fig. 5).

3 Conversion of Lignocellulosic Biomass

Native lignocellulosic materials are heterogeneous, composed of cellulose and hemicellulose as the carbohydrate components. Table 1 shows the approximate composition of the selected lignocellulosic materials. Cellulose is a high molecular weight linear polysaccharide. The degree of polymerization of cellulose ranges from 200 to 2000 kDa, while hemicellulose is a rather low molecular weight heteropolysaccharide with a degree of polymerization less than 200. Unlike the orderly structure of cellulose, hemicellulose shows a wide variation

Table 1. Approximate composition of selected cellulosic materials

Materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Herbaceous			
Alfalfa hay	38	9	14
CBG[a]	25	35.7	6.4
Switchgrass	45	31	12
Crop residues			
Corn cobs	45	35	15
Corn stover	41	21	17
Wheat straw	36	28	29
Hardwood			
Aspen	46	26	18
Hydrid poplar	43	21	26
Softwood			
Spruce	43	26	29
Pine	44	26	29
Cellulose wastes			
Newsprint	61	16	21
RPS[b]	50	10	n/a

[a] Coastal Burmuda grass.
[b] Recycled paper sludge.

in both structure and constitution. The interior chain of hemicellulose consists of polysaccharides that are often attached to a variety of sugar moieties that are the same or different from the sugars that form the side chains. These types of hemicelluloses are often classified according to the sugar residues present. Commonly occurring hemicelluloses are xylan, arabino-xylan, gluco-mannan, galacto-gluco-mannan, and others. L-Arabinan is often associated with pectic material but is usually considered to be a hemicellulose. The structure of most hemicelluloses is β-1,4-linked, except for the galactose-based hemicelluloses, which are β-1,3-linked. The detailed structure and composition of hemicellulose have been reviewed [19].

Processes for the bioproduction of ethanol from cellulosic materials have been studied extensively. Some of the process steps are specialized and beyond the scope of this chapter. However, there are many recent review articles dealing with some specific subjects. Basically, the processes consist of a number of steps. They are: availability and collection of raw feedstock [20], size reduction, pretreatment, fractionation of biomass components, enzyme production [21, 22], saccharification, enzyme recycle [23, 24], pentose fermentation, improvement of pentose-fermenting biocatalyst, overcoming of product inhibition, overcoming inhibition by substrate-derived inhibitors, ethanol recovery [25], steam generation and recycling [26], waste treatment, and by-product utilization.

3.1
Biomass Pretreatment

Many factors influence the reactivity and digestibility of the cellulose fractions of lignocellulose materials. These factors include lignin and hemicellulose content, crystallinity of cellulose, and the porosity of the biomass materials. Pretreatment of lignocellulosic materials prior to utilization is a necessary element in biomass-to-ethanol conversion processes. The objective of the pretreatment is to render biomass materials more accessible to either chemical or enzymatic hydrolysis for efficient product generation. The goals of the pretreatment are:

(1) to remove and separate hemicellulose from cellulose,
(2) to disrupt and remove the lignin sheath,
(3) to decrease the crystallinity of cellulose,
(4) to increase the accessible surface area of cellulose, and
(5) to increase the pore size of cellulose to facilitate the penetration of hydrolysis agents.

There have been many processes developed for the treatment of various biomass materials. Normally, agricultural residues are more easily treated than hardwood materials. The most difficult substrate for treatment is softwood materials because they have a relatively high lignin content. Most processes exploit a variety of mechanisms in order to render the carbohydrate components of lignocellulosic materials more susceptible to enzymatic hydrolysis and microbial conversion (for details, see [27–30]).

The majority of pretreatment methods involve a combination of mechanical size reduction, alkali swelling, acid hydrolysis, steam and other fiber explosion techniques, or exposure to supercritical fluids. The processes involving irradiation, strong mineral acids, cellulose solvents, and concentrated alkali chemicals are not suitable methods for the pretreatment of lignocellulosic materials due to their high cost. Biological methods such as those applying "white rot" fungi to remove lignin from cellulose-hemicellulose are also unsuitable due to the length of time involved, unless, that is, the lignocellulosic material is intended for single-cell protein (e.g., mushrooms) production [31]. Therefore, the chosen pretreatment process must be efficient, cost effective, and environmentally friendly.

Many different pretreatment approaches have been designed and tested, and some processes have also been tried on a pilot scale. Ideally, the most desirable method of treatment is the dissolution of the solid materials into an aqueous substrate. Many cellulose solvents at high enough concentrations can penetrate into the cellulose crystal structure causing the dissolution of solid cellulose. The resulting material can be readily hydrolyzed to glucose by cellulase either in situ, or after the dissolved cellulose has been reprecipitated [32]. Although cellulose solvents such as zinc chloride are powerful cellulose solubilization agents, they are either toxic, corrosive, hazardous, or expensive. For these reasons, cellulose solvents are generally considered inappropriate for large-scale pretreatment processes.

3.1.1
Alkaline Treatment

Alkaline treatment causes lignocellulosic materials to swell; increased swelling leads to higher susceptibility of cellulose to saccharification. In the presence of alkaline chemicals (e.g., NaOH or NH_3), cellulose, hemicellulose, and lignin bonds can be disrupted. This permits cellulose to swell beyond normal water-swollen stages. Consequently, the pore size, the intraparticle porosity, and the capillary size are increased. There is also a phase change in the cellulose crystalline structure [33].

Sodium hydroxide is the most commonly used chemical in the treatment of lignocellulose to enhance digestibility for animals. It is a strong swelling agent for cellulose and may also be responsible for changing the cellulose crystalline structure from cellulose I to cellulose III (decrystallization). The amount of NaOH used for treatment ranges from 2 to 20%, and the temperature for the treatment ranges from ambient to 120°C. Under mild conditions (e.g., low alkaline concentration and low temperature) substrate components remain unchanged, while under harsher conditions most of the lignin and hemicellulose are solubilized. Consequently, the cellulose fraction is exposed to hydrolysis agents.

Similar to NaOH, ammonia is useful in increasing the in vitro digestibility of lignocellulosic materials. It is also effective in the preparation of substrates for single-cell protein production. Ammonia is one of the most heavily used industrial commodity chemicals. Based on weight, aqueous ammonia is about one-third the cost of NaOH. In addition, ammonia is easily recoverable from an aqueous mixture because of its high volatility; it is also far less corrosive than mineral acids at a high treatment temperature. Increasingly, ammonia has been used in combination with other methods to treat lignocellulosic biomass.

3.1.2
Steam Treatment With or Without SO_2

Biomass material (woodchips or wheat straw) is placed in a high-pressure stainless steel tube and exposed to steam under pressures ranging from 250–650 psi at 200 to 240°C for up to 20 min. The sudden pressure release causes an explosion of biomass material thereby disrupting the lignin and hemicellulose bonding toward cellulose. Many investigators (see [29] for review) have studied the steam explosion of biomass materials. The addition of SO_2 enhances the pretreatment effect and also increases the recovery of hemicellulose [34–36]. A refinement of this pretreatment method has recently been reported by Stenberg et al. [37].

3.1.3
Ammonia Fiber Explosion (AFEX)

AFEX is the pretreatment method that utilizes steam explosion techniques using ammonia as the chemical reagent [38–41]. The intended biomass material is placed in a pressure vessel with liquid ammonia (1:1 basis) and treated

as in the steam explosion process with much lower temperatures (up to 90°C) and pressures (up to 15 psi) for 15 to 30 min. Upon the release of pressure, fibrous biomass is exploded to create the pore size and the released ammonia is then recovered for reuse. Exposure to ammonia in the AFEX process causes swelling and partial decrystallization of the cellulose. It also causes the partial solubilization of lignin and hemicellulose resulting in the exposure of cellulose. The resulting treated cellulosic materials have proved to be more susceptible to cellulolysis by cellulases. This pretreatment method is more effective with agricultural residues (e.g., corn stover and straws) than with woody materials.

3.1.4
Ammonia-Recycled Percolation Process

The ammonia-recycled percolation process [42,43] uses ammonia as the treatment agent. The reactor is a packed-bed flow-through-type reactor (percolation reactor) and is used in a recirculation mode. The intended biomass material is packed into a pressure vessel with preheated liquid ammonia passing through the substrate. The reaction temperature is up to 180°C, and pressures are up to 325 psi. As the name of the process indicates ammonia is recycled for further treatment of cellulosic materials. A scanning electron microscope examination of the treated samples indicates an increase in pore size and porosity. The amount of lignin removed is in the range 23–63%, and hemicellulose removed in the range 20–36%. This pretreatment method is more effective with agricultural residues (e.g., corn stover and straws) than with woody materials. The resulting glucan can be hydrolyzed by cellulase to near completion.

3.1.5
Alkaline Peroxide

Chopped wheat straw is steeped in an aqueous alkaline solution (pH 11.5) of H_2O_2 at a ratio of 0.25 g/g substrate at 25°C. Lignin removal is 50%, and hemicellulose solubilized is almost complete. The results of *Trichoderma* cellulase hydrolysis of cellulose indicates that the glucose yield approaches 100% with a substrate solid content of 5% [44]. Patel and Bhatt [45] used this method to remove lignin from rice straw. Similarly, Kim and Lee [46] used this method to pretreat corn cob/corn stover mixture and switchgrass. In their study, ammonia (10 wt%) and hydrogen peroxide (0.28 g H_2O_2/g biomass) were pumped simultaneously into a packed-bed flow-through-type reactor (percolation reactor) at 170°C in a semibatch mode with ammonia recycle. Delignification achieved was 94–99% and about 80% total hemicellulose was solubilized to xylose oligomers. After treatment, the remaining solid had the following composition: glucan: 80–93%; xylan: 5–10% and lignin: 1–6%.

3.1.6
Lime Pretreatment

Chang et al. [47] used calcium hydroxide as the pretreatment agent to enhance the enzymatic digestibility of switchgrass. The optimum pretreatment condi-

tions were as follows: lime loading: 0.1 g $Ca(OH)_2$/g dry biomass; water loading: 9 ml/g dry biomass; temperature: 100 – 120 °C and reaction time: 2 h. After treatment, the enzymatic digestibility (3 d) was enhanced by 7 times for glucose yield from cellulose and 21 times for xylose yield from hemicellulose. During pretreatment, about 10 % of glucan, 26 % of xylan, and 29 % of lignin were solubilized.

3.1.7 *Dilute Acid Pretreatment*

The use of dilute acid hydrolysis of hemicellulose as a pretreatment method has been studied extensively (see [27,28] for reviews). The overall goal of dilute acid pretreatment is to attain a high yield of hemicellulose carbohydrates while minimizing the breakdown of hemicellulose sugars into decomposition products. The pretreatment usually involves mineral acids (e. g., HCl and H_2SO_4) at a concentration of 0.3 to 2 % (w/w). The temperature for treatment ranges from 120 to 180 °C and the length of treatment from less than 1 min to 2 h or longer. The objective of dilute acid treatment is to separate hemicellulose from lignin and cellulose. While it is effective in hydrolyzing hemicellulose, a portion of lignin is also solubilized resulting in complication in the utilization of hemicellulose carbohydrates. Depending on the hydrolysis conditions, carbohydrate degradation products can form that interfere with microbial activities.

Because the hemicellulose fraction of biomass materials can be separated from lignin and cellulose by dilute acid treatment, cellulose becomes more reactive towards cellulase. Hemicellulose hydrolysis rates vary with acid concentration, temperature, and solid-to-liquid ratio. With most lignocellulosic materials, complete hemicellulose hydrolysis can be achieved in 5 – 10 min at 160 °C or 30 – 60 min at 140 °C. Dilute acid hydrolysis forms the basis of many pretreatment processes; for example, autohydrolysis and steam explosion are based on high-temperature dilute acid catalyzed hydrolysis of biomass.

Torget et al. [48] pretreated hardwoods (silver maple, sycamore and black locust) and corn residues (cobs and stover) with dilute H_2SO_4 (0.45 – 0.5%) at 140 and 160 °C. After pretreatment, more than 90 % of the cellulose was hydrolyzed by cellulase. Furthermore, Torget and Hsu [49] applied a two-temperature dilute H_2SO_4 hydrolysis of hybrid poplar using a percolation process. In a more recent study, Torget et al. [50] optimized the conditions of a reverse-flow, two-temperature dilute H_2SO_4 pretreatment of yellow poplar sawdust. In this study, lower temperatures (150 – 174 °C) were applied to hydrolyze the easily hydrolyzable xylan, and higher temperatures (180 – 240 °C) were applied to hydrolyze the remaining xylan. As much as 97 % of xylan was recovered with only 2.9 % of xylose being degraded to furfural. The resulting solid (mainly cellulose) was readily converted to ethanol via the simultaneous saccharification and fermentation (SSF) process. Nguyen [51] soaked Douglas fir chips in a 0.4 % H_2SO_4 solution at 60 °C for 4 h then pretreated the material at 200 – 230 °C for 1 – 5 min. After pretreatment, 90–95% of the hemicellulose and as much as 20 % of the cellulose was solubilized in aqueous solution. Of the remaining cellulose, 90 % can be hydrolyzed by cellulase to glucose.

Dilute hydrochloric acid is as effective in the hydrolysis of the hemicellulose components of lignocellulosic biomass as sulfuric acid but it is more corrosive to equipment. Other acids such as dilute phosphoric acid have been used to hydrolyze sugar cane bagasse hemicellulose [52].

3.1.8
Aqueous (Liquid Hot Water) Pretreatment

According to Kohlmann et al. [53], the objective of aqueous pretreatment of lignocellulose is to hydrate the crystalline structure and to modify it to a form that is more accessible and susceptible to enzyme hydrolysis. With this pretreatment method, biomass materials are saturated with water and placed in a reactor followed by heat treatment between 220 and 240 °C. Mok and Antal [54] pretreated different biomass materials with compressed hot water at 200 – 230 °C for up to 15 min. In all cases, hemicellulose was completely solubilized. In addition, up to 45 % of lignin and up to 20 % of cellulose were solubilized. Van Walsum et al. [55] used a custom-built 250-ml immersed percolation reactor with a single-stage processing of 10 – 15 g of sugar cane bagasse and hardwood materials at 220 °C for 2 min. In the case of aspen, a three-single-stage process was employed consecutively. Recently, Weil et al. [56] gave a detailed description of this pretreatment method as well as the reactor system used to carry out pretreatment of yellow poplar sawdust.

4
Biomass Fractionation

The rationale behind the fractionation of lignocellulose into lignin, hemicellulose and cellulose components prior to utilization is as follows:

(1) lignin and hemicellulose form a sheath over the cellulose fiber,
(2) alkaline extractives, soluble lignin, and lignin-derived soluble products inhibit microbial fermentation,
(3) hemicellulose is much more susceptible to acid hydrolysis than cellulose, and
(4) cellulose carbohydrate (glucose) can be fermented to ethanol much faster than hemicellulose-derived carbohydrates (e. g., xylose).

The biomass fractionation options and their economical benefits have been discussed by Elander and Hsu [57]. The objective of the fractionation is to separate different biomass components and subsequently convert the cellulose and the hemicellulose into products separately. To achieve this objective, mild treatment conditions, such as low temperature, low concentration of reagents (or easily recoverable reagents) and low pressures, are necessary. Under this biomass component fractionation scheme, cellulose sugar (glucose) and hemicellulose carbohydrates (mostly xylose) can be converted into products in different reactors to produce different products if so desired.

Carrasco et al. [58] analyzed the effect of dilute H_2SO_4 and steam explosion pretreatment on the fractionation and kinetics of several biomass materials.

Cao et al. [59] used zinc chloride solution (64 wt%) to dissolve wheat straw prior to fractionating the biomass components. With this method, hemicellulose was hydrolyzed to its sugar constituents by heating at 60°C. Cellulose and lignin were recovered as the precipitates upon the addition of water to adjust the solution to 35% zinc chloride concentration. Cellulose was further hydrolyzed by cellulase to glucose.

Cao et al. [60–62] examined a fractionation option that used corn cob and aspen woodchip as the substrates. In this biomass fractionation scheme (Fig. 6), the majority of lignin, alkaline extractives, and acetate were solubilized and separated from cellulose and hemicellulose fractions by alkaline treatment. Hemicellulose was then hydrolyzed to its sugar constituents with dilute acid (0.3 M HCl). Hemicellulose carbohydrates were then fermented to ethanol by a xylose-fermenting yeast strain (Fig. 7). The cellulose fraction, after separation from lignin and hemicellulose, was used as the substrate in the SSF process for ethanol production using a thermotolerant yeast strain as the biocatalyst (Fig. 8).

Wu and Lee [63] fractionated switchgrass into hemicellulose, cellulose and lignin. Under their fractionation scheme, the hemicellulose fraction was

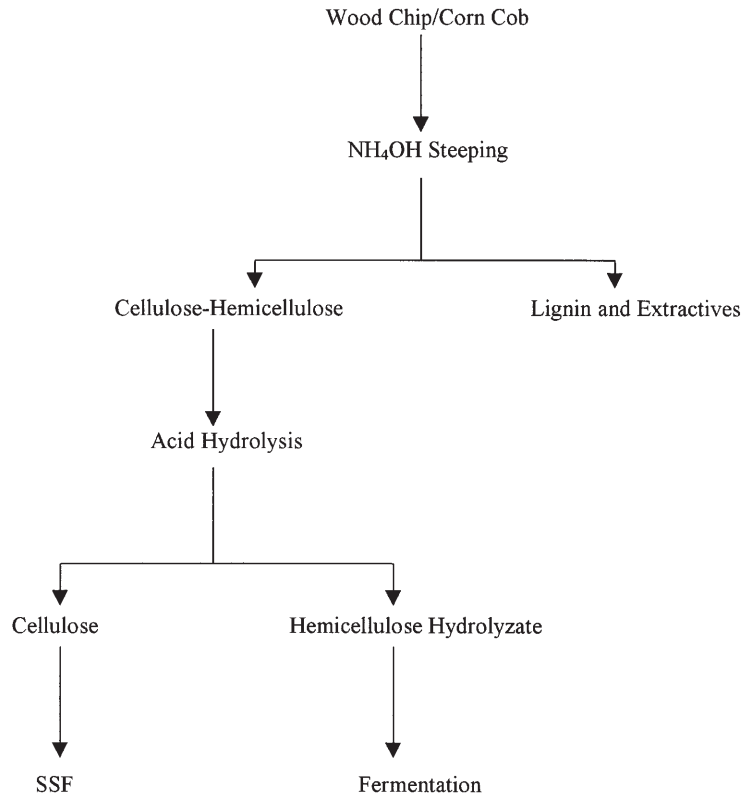

Fig. 6. Flow chart of biomass fractionation [60]

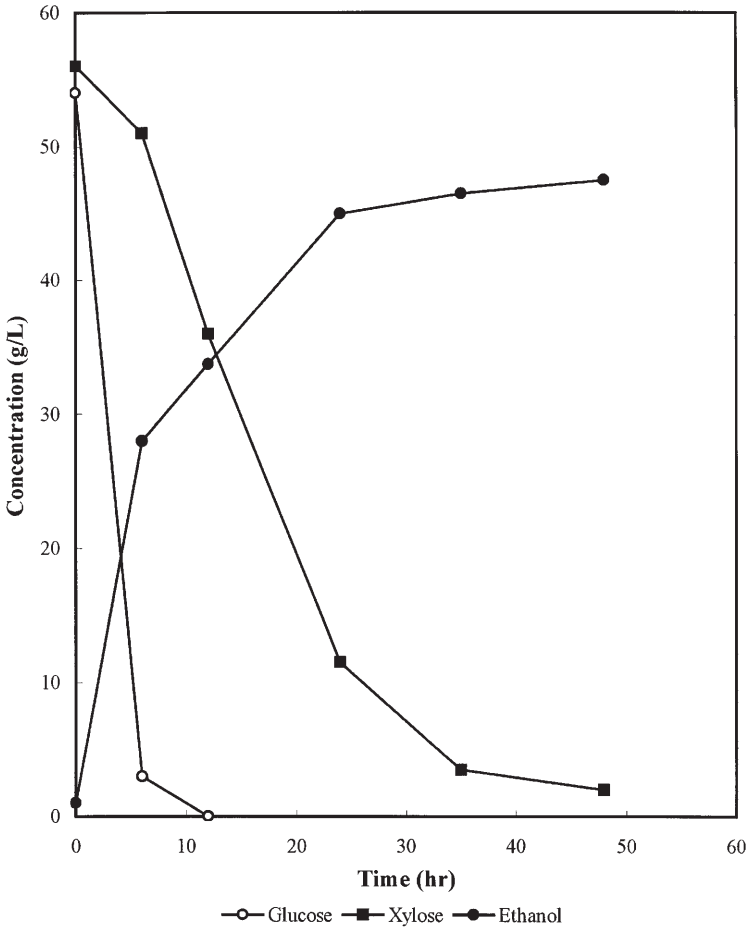

Fig. 7. Fermentation of hemicellulose-cellulose hydrolysate to ethanol from corn cob using *Saccharomyces* 1400 (pLNH33) [61]

completely solubilized by dilute sulfuric acid (0.078 wt%) at 145–170°C. The solid residue with 70% glucan and 30% lignin was then subjected to the ammonia-recycled percolation process to remove lignin. The resulting residue of the combined pretreatment contained about 90% glucan and 10% lignin. The digestibility of this residue was much higher and required less cellulase loading. Likewise, Schell et al. [64] treated Douglas fir by acid-catalyzed steam explosion followed by alkali treatment to fractionate the material into different components.

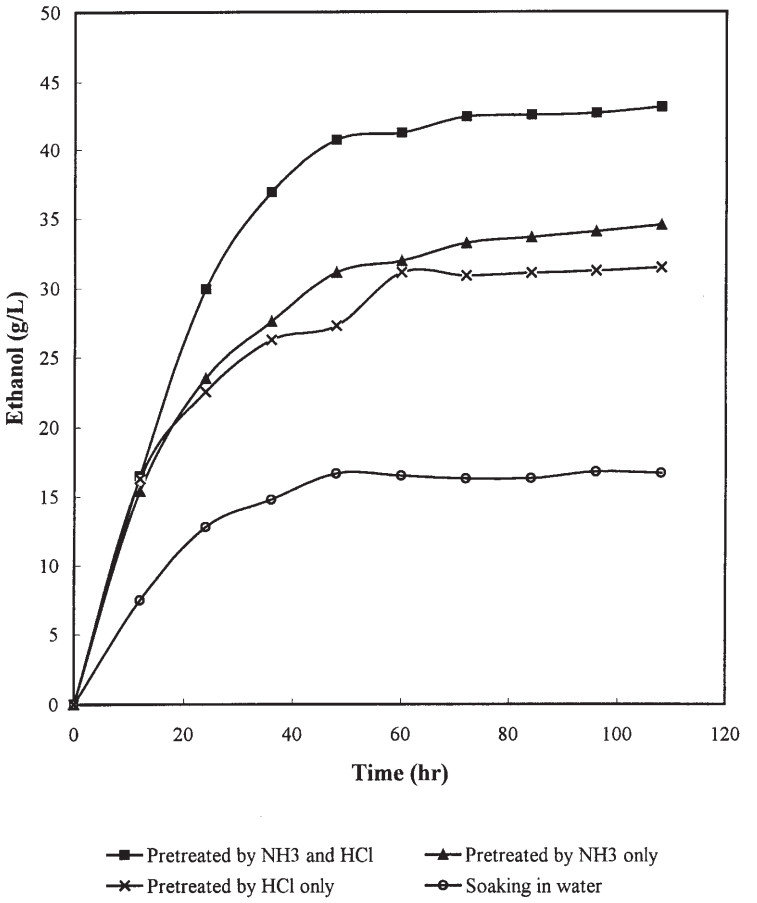

Fig. 8. SSF of corn cob for ethanol production after different treatment [61]

5 Simultaneous Saccharification and Fermentation (SSF)

SSF is a process in which the production of ethanol from cellulosic materials is achieved by utilizing cellulose, cellulase, ethanol-producing microbes and nutrients in the same reactor. This process is desirable because the continuous removal of sugars by fermentative organisms alleviates end-product inhibition of enzyme hydrolysis of cellulose. The process is also simplified because only one reactor is used. The SSF process for ethanol production from cellulosic materials was reported by Blotkamp et al. [65] and was later tested on a pilot scale (for detail, see [66]).

The following are the advantages of SSF over the separate saccharification and fermentation of cellulose:

(1) reduction in contamination risk,
(2) lower enzyme loading required,
(3) faster hydrolysis reaction rate,
(4) gives higher product yield, and
(5) lower operational costs.

For these reasons, SSF is the preferred process option for the bioconversion of the cellulose fraction of lignocellulosic materials to ethanol. The SSF process involving lignocellulosic materials has been reviewed [66]. The following factors are important for an efficient SSF process:

(1) the physical state of the substrate (particle size and crystallinity of cellulose),
(2) characteristics of the cellulase and enzyme loading,
(3) effect of fermentation product on enzyme activity,
(4) compatibility of saccharification and fermentation temperature,
(5) characteristics of the yeast strains and yeast concentration, and
(6) maintainance of low levels of products.

The nature of the cellulosic substrate is important because the degree of saccharification in the SSF system is often rate-limiting with respect to ethanol production. Temperature also determines the economics of ethanol production from cellulose. In their study of the limiting factors in the SSF of cellulosic biomass to ethanol, Philippidis and Smith [67] identified the accessibility of cellulose as the key factor determining the rate of SSF. During the early stages of SSF, cell growth is the rate-limiting step. As the fermentation proceeds, enzymatic hydrolysis becomes the limiting factor. In most studies, cell densities and enzyme concentration exerted little effect on the amounts of ethanol produced from cellulose under typical SSF conditions.

5.1
Supraoptimal Temperature Fermentation

In designing an efficient SSF system for the conversion of cellulose to ethanol, the fermentation temperature should be compatible with the saccharification temperature that is generally between 45 and 55°C. The optimal temperature for the most commonly available cellulase is about 50°C. Therefore, the use of high-temperature-tolerant microbes is desirable for the application of the SSF process to ethanol production. Typical industrial ethanol-producing yeast strains are mesophilic with an optimal fermentation temperature of 30–37°C. Only a few yeast strains that are thermotolerant, as well as good ethanol fermentors, have been described. However, some thermophilic bacterial species are known to produce ethanol from cellulosic-derived carbohydrates [68,69].

5.1.1
Thermotolerant Yeasts

Anderson et al. [70] studied several thermotolerant yeast strains isolated from sugar cane factories in Australia. Most of the good ethanol-producing yeasts be-

longs to *Kluyveromyces marxianus var. maxianus*. Some are capable of producing over 6% ethanol (w/v) from glucose at an incubation temperature of up to 43°C after 14 h of fermentation. Over 80% of yeast cells remained viable at the end of fermentation. Similarly, Banat et al. [71] isolated several thermotolerant, alcohol-producing yeast cultures from India. These are capable of producing up to 70 g/l ethanol at 45°C and up to 55 g/l ethanol at 50°C.

Ballesteros et al. [72] studied SSF of pure cellulose with *K. maxianus* and *K. fragilis* at 45°C with cellulase loading of 15 FPU/g substrate. Both yeast strains produced close to 38 g/l ethanol in 78 h. The results also confirmed the importance of using thermotolerant yeast in SSF processes in order to improve hydrolysis rates and achieve higher ethanol production. Possible benefits of using a thermotolerant yeast to carry out ethanol fermentation at a supraoptimal temperature are:

(1) faster rates of saccharification,
(2) higher rates of sugar utilization and product (ethanol) formation,
(3) reduction of contamination by mesophilic yeast and bacteria,
(4) facile product recovery, and
(5) simple cooling during industrial-scale fermentation due to the exothermal fermentation.

However, yeast activity is more sensitive to the inhibitory effects of ethanol as the temperature is increased.

5.2 Improvement of the SSF Process

Typically, the SSF process is carried out in a CSTR reactor in batch mode. Under these reaction conditions, the fermentation product, ethanol, exerts its effect not only on microbes but also on saccharification. To overcome this problem, and to improve the efficiency of ethanol production from cellulose, the continuous removal of end-product during ethanol production would have advantages. With this type of process application, the SSF process can be operated in a fed-batch mode. Fed-batch operation is similar to continuous operation except the fermentation broth is retained in the fermentor at all times whereas the solid substrate is continuously fed into the fermentor [73]. Another method is to continuously remove ethanol during the SSF process (see Sect. 2.1.3).

6 Hemicellulose Fermentation

When hemicelluloses are hydrolyzed, a mixture of monosaccharides is produced. The predominant sugars released are xylose, glucose and, to a lesser extent, arabinose, galactose and mannose. When microorganisms are exposed to this sugar mixture, the phenomena of diauxic growth and differential rates of sugar utilization are often observed. The differential utilization of sugars by a given microorganism is dictated by sugar uptake, utilization rates, and by the degree of catabolic repression that affects specific enzyme activity and biosynthesis. As

a rule, microorganisms prefer glucose over galactose followed by xylose and arabinose. Usually, hexoses in the hydrolysate can be completely fermented to ethanol within a few hours. However, the complete conversion of xylose to ethanol will take 48 to 72 h or longer. The non-matching fermentation rates between glucose and xylose cause difficult design problems. The development of the SSF process has made it possible to convert lignocellulosics to ethanol with cellulase and fermenting microbes in the reactor at the same time. The conversion of cellulose to glucose and finally to ethanol can be completed quickly. Nevertheless, the batch lasts until the xylose fermentation is complete.

6.1 Fermentation Inhibitors

In addition to producing monomer sugar, thermochemical processing of biomass is known to produce substances that inhibit microbial growth and ethanol production [74,75]. For example, furfural derived from pentose and hydroxymethylfurfural derived from hexose are known inhibitors [75] (Table 2). In addition, soluble phenolic compounds derived from lignin degradation and alkaline extractives are also inhibitory towards yeast in low concentration. Another potential inhibitor is acetic acid that can be present in relatively high concentrations (6–15 g/l); it exists as an integral part of hemicellulose. Various procedures have been investigated to minimize the inhibitory effect of the processing by-products. These methods include the following: carefully control of the pretreatment process to minimize sugar degradation, use of ion-exchange resins and activated charcoal to remove the inhibitors, soaking the materials in alkaline solution prior to hydrolysis and the adaptation of microorganisms to substrate with inhibitors [76].

6.2 Xylose Fermentation

Xylose can be metabolized by bacteria, fungi or yeast. In bacteria, the initial step of xylose metabolism involves inducible enzymes (i.e., xylose transport enzymes, xylose isomerase and xylulokinase). The direct isomerization of xylose

Table 2. Effect of inhibitors on ethanol production from glucose and xylose [75]

Compound	Concentration (g/l)	% Inhibition (EtOH)	
		Glucose[a]	Xylose[b]
Furfural	2	89	95
HMF[c]	5	95	91.4
Acetate	10	27	69

[a] *S. cerevisiae*.
[b] *P. stipitis*.
[c] Hydroxymethylfurfural.

to xylulose is the first step in xylose utilization by bacteria. They produce a variety of end-products including ethanol from xylose [77]. Recently, through advances in genetic engineering, a few bacterial strains have been genetically constructed to efficiently produce ethanol as the major product from xylose (see e.g., [78,79]).

In yeast and mycelial fungi, xylose is metabolized via "coupled oxidation-reduction reactions". Xylose reductase is the enzyme involved in the reduction of xylose to xylitol. Sequential enzymatic events, through the oxidation of xylitol to xylulose, lead to the utilization of xylose. Many yeast species utilize xylose readily, but the ethanol production capability is very limited. Only a few yeast species effectively produce ethanol from xylose; these include *Pachysolen tannophilus, Candida shihatae* and *Pichia stipitis* [80]. The production of ethanol from xylose by these three yeast strains has been studied extensively in recent years. Recently, genetically engineered yeast strains have been constructed for more effective conversion of xylose to ethanol.

6.2.1 *Conversion of Xylose to Ethanol by Yeasts*

Although many facultatively fermentative yeasts utilize xylose as the carbon source for growth, the ability of these yeasts to produce ethanol from xylose is limited. Yeast strains that utilize xylose often produce xylitol from xylose extracellularly as a normal metabolic activity. However, only a few can produce significant quantities of ethanol. The prominent strains that produce ethanol from xylose include: *Pachysolen tannophilus*, *Candida shihatae* and *Pichia stipitis*. However, the efficient production of ethanol from xylose is limited by the regulation of dissolved oxygen as well as by the imbalance of cofactors in the metabolic pathway during xylose utilization. In recent years, much effort has been put into improving yeast strains in order to produce ethanol from xylose more efficiently.

6.2.1.1 *Metabolic Pathway*

Ethanol fermentation from xylose by yeasts can be divided into four distinctive steps. The first step is the reduction of xylose to xylitol mediated by NADPH/NADH-linked xylose reductase (XR). This is followed by the oxidation of xylitol to xylulose, mediated by NAD-linked xylitol dehydrogenase (XDH). Xylulose-5-phosphate, the key intermediate, is generated from the phosphorylation of xylulose by xylulose kinase. Xylulose-5-phosphate is then channeled into the pentose phosphate pathway for further metabolism (Fig. 9).

6.2.1.2 *Ethanol Yield and Carbon Balance*

Ten molecules of ethanol can be produced from six molecules of xylose by using a combination of fermentative and pentose phosphate pathways. The net

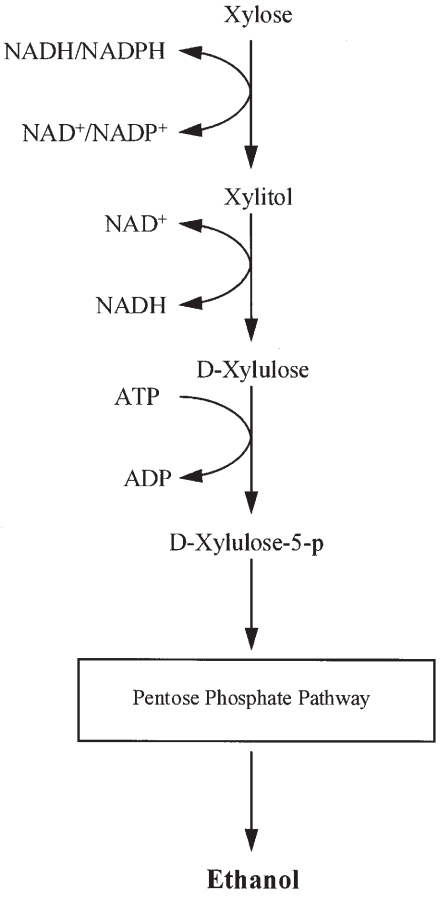

Fig. 9. Abbreviated pathways for xylose utilization by yeasts

equation for the reactions leading to the production of ethanol from xylose is as follows:

$$6 \text{ Xylose} \rightarrow 10 \text{ Ethanol} + 10 \text{ CO}_2$$

These values are the theoretical maximum yields (same as glucose fermentation) that represent the maximum value obtainable. This can be achieved only if no sugar is assimilated to cell mass or oxidized through the TCA cycle. Of the total carbon contained in xylose, two-thirds goes to ethanol and the other one-third is lost as CO_2. On a weight basis, the yield of ethanol from both xylose and glucose is approximately 51%.

6.2.1.3
General Characteristics of Yeasts That Ferment Xylose to Ethanol

(1) Oxygen requirement [81]:
- Oxygen is required for the efficient uptake of xylose.
- Under low dissolved oxygen (DO) conditions, the electron transport system is not able to oxidize NADH efficiently. This causes an imbalance of $NADH/NAD^+$ that leads to the accumulation of xylitol.
- An increase in DO will enhance cell growth and xylose fermentation.
- When oxygen is in excess, TCA cycle activity will be enhanced which results in excessive cell growth and causes the reassimilation of ethanol produced.
- The reassimilation of ethanol leads to the accumulation of acetaldehyde and acetic acid.
- Optimal levels of DO will give the lowest xylitol accumulation with the highest ethanol yield.

(2) Enzyme cofactor imbalance:
- Xylose reductase and xylitol dehydrogenase have different cofactor requirements.
- Different nitrogen source (organic vs. inorganic) affects the level of xylitol dehydrogenase activity [82].
- Different DO affects the balance of cofactors.

(3) Low ethanol tolerance:
- Fermentation will be inhibited at an ethanol concentration of 42 to 45 g/l.

(4) Xylitol, glycerol, arabitol and acetate are the common by-products.

(5) Higher sensitivity to fermentation inhibitors than glucose-fermenting yeast strains (see Table 4).

(6) Specific ethanol productivity from xylose is at least one order of magnitude lower than that in glucose fermentation by *S. cerevisiae*.

6.2.1.4
Natural Yeast Strains

Pachysolen tannophilus. *P. tannophilus* was originally isolated by Wickerham [83] from wood sulfite liquor. This yeast was the first naturally occurring yeast species that was found to produce significant quantities of ethanol from xylose [84]. A more detailed study of this yeast was reported by Slininger et al. [85]. According to the reported literature, ethanol production from xylose by *P. tannophilus* exhibits the following characteristics:

(1) A lag phase of about 20 h is observed before the accumulation of ethanol.

(2) Ethanol yield is reported to be 0.34 g ethanol/g xylose consumed (68% of the theoretical value).

(3) Optimal pH for cell growth is 2.5–4.5 and optimal pH for ethanol production is 2.5.

(4) Ethanol production is subject to substrate (55 g/l xylose) and product (30 g/l ethanol) inhibition.

(5) Xylitol is accumulated during fermentation.

Candida shehatae. The ability of *C. shehatae* to produce ethanol from xylose was first reported by du Preez and van der Walt [86] in 1983. Since then, many studies have been conducted related to the property of this yeast. According to the reported literature [87–89], ethanol production from xylose by *C. shehatae* exhibits the following characteristics:

(1) A small amount of dissolved oxygen is required for maximal ethanol production.
(2) Cell growth is inhibited at an ethanol concentration of 37.5 g/l, and cell viability is significantly lower in the presence of up to 50 g/l ethanol.
(3) Ethanol yield can be as high as 84% of the theoretical value (0.43 g ethanol/g xylose consumed).
(4) The maximum ethanol production rate is 0.48 g/g/h at 50 g/l xylose.
(5) Xylitol is accumulated under a very limited dissolved oxygen condition due to the accumulation of NADH.

Pichia stipitis. *P. stipitis* is the most effective natural yeast for the conversion of xylose to ethanol. This yeast species shares many characteristics with its close relative, *C. shehatae*. Toivola et al. [90] performed a systemic screening program with type strains of some 200 yeast species and identified *P. stipitis* as one of the yeast species that produces ethanol from xylose. There are many studies that have explored the property of this yeast in relation to its oxygen requirement, ethanol tolerance, enzyme cofactor's balance, etc. According to the reported literature [91,92], ethanol production from xylose by *P. stipitis* exhibits the following characteristics:

(1) Trace oxygen is required to sustain cell growth and maintenance.
(2) *P. stipitis* has a higher ethanol (64 g/l) tolerance than any other xylose-fermenting yeast.
(3) Compared to *C. shehatae*, *P. stipitis* has a lower specific ethanol productivity with a higher ethanol yield.
(4) Up to 57 g/l of ethanol can be accumulated by *P. stipitis*.
(5) Xylitol production by *P. stipitis* is much lower than any other xylose-fermenting yeast.

6.2.1.5 *Genetically Modified Yeasts*

Attempts to modify the xylose-fermenting pathway in *S. cerevisiae* using xylose isomerase genes from various bacterial sources have not been successful [93]. This is in spite of previous reports that showed the ability of glucose-fermenting yeast strains, *S. cerevisiae* [94] and *Schizosaccharomyces pombe* [95], to produce ethanol from xylulose or xylose in the presence of xylose isomerase (glucose isomerase) [96]. Other approaches to improving yeast strains through genetic recombination have met with some encouraging results.

Saccharomyces cerevisiae. Kotter and Ciriacy [97] studied xylose utilization of an *S. cerevisiae* transformant that expressed two key enzymes (xylose reductase

and xylitol dehydrogenase) derived from *P. stipitis* in xylose metabolism. Under fermentative conditions, the transformant converts only half of available xylose to xylitol and ethanol. The acquired ability to ferment xylose was interpreted as a result of the dual cofactor dependence of the XR and the generation of NADPH by the pentose phosphate pathway. The limitation of xylose utilization in the transformant was more likely caused by an insufficient capacity of the nonoxidative pentose pathway. This was indicated by the accumulation of sedoheptulose-7-phosphate and the absence of fructose-1,6-diphosphate and pyruvate accumulation.

Saccharomyces 1400 (pLNH33). This recombinant yeast strain was developed using a high-ethanol-tolerant yeast *Saccharomyces* 1400 [4,5] as the host. It was cloned with xylose reductase and xylitol dehydrogenase genes from *P. stipitis* and the amplification of the xylulokinase gene. *Saccharomyces* 1400 (pLNH33) was shown to ferment both sugars in a 1:1 mixture of glucose (52.8 g/l) and xylose (56.3 g/l) to ethanol under microaerobic conditions in a relatively high yield (84% of theoretical value) [98]. A final ethanol concentration of 48 g/l was obtained after 48 h of incubation. This recombinant was also shown to produce ethanol from glucose and xylose that were derived from corn fiber during a simultaneous saccharification and fermentation process in the presence of a fungal cellulase [99].

6.2.2
Conversion of L-Arabinose to Ethanol by Yeasts

Little attention has been focused on the fermentation of L-arabinose to ethanol. Recently, McMillan and Boynton [100] evaluated eight fungal and six yeast strains for ethanol production from arabinose under oxygen-limited conditions. None of the strains tested produced ethanol from arabinose. They utilized arabinose for cell biomass and L-arabitol production.

6.2.3
Conversion of Xylose to Ethanol by Bacteria

A wide range of bacterial species utilizes D-xylose and L-arabinose as carbon and energy sources. In most cases, the direct isomerization of aldopentoses to their corresponding ketoses is the first step in pentose metabolism. For example, D-xylose is converted into D-xylulose and L-arabinose is converted into L-ribulose; L-ribulose can be converted into D-xylulose by epimerase. D-Xylulose is the key intermediate for further metabolism. For this reason, most bacterial species can also readily utilize L-arabinose.

Bacterial species, particularly those belonging to *Klebsiellae*, *Erwinia* and *E. coli*, are known for their ability to metabolize hexoses and pentoses to produce either neutral compounds (butanediol, acetoin and ethanol) or mixed acids and ethanol under specific cultural conditions. Research on the production of ethanol from pentoses by bacteria has revolved around the improvement of such bacteria through genetic recombination.

6.2.3.1
Klebsiella oxytoca M5A1

Pyruvate decarboxylase and alcohol dehydrogenase genes encoding the ethanol pathway from *Z. mobilis* were transferred into *K. oxytoca* [101]. The transformant, *K. oxytoca* M5A1, was able to divert pyruvate from the normal fermentative pathway to ethanol production. *K. oxytoca* M5A1 was able to produce ethanol from both glucose and xylose in excess of 40 g/l with an efficiency of 0.48 g of ethanol/g xylose and 0.5 g of ethanol/g glucose. The maximal volumetric productivity for both sugars was similar (ca. 2 g/l).

6.2.3.2
Klebsiella planticola

Multicopy plasmids containing the *Z. mobilis* pyruvate decarboxylase gene was inserted into *K. planticola*. The transformant was shown to produce 31.6 g/l ethanol from a mixture of sugars (79.6 g/l) that included xylose [102]. The yield of acids (formic, acetic, lactic) and butanediol was reduced significantly.

6.2.3.3
Escherichia coli

Pyruvate decarboxylase of *Z. mobilis* was transferred into *E coli* [103]. The transformant was able to produce 39.2 g/l ethanol from 80 g/l xylose with an indicated yield of 96% of the theoretical value. The maximal volumetric productivity was 0.7 g/l ethanol per hour in batch fermentation [104].

6.2.3.4
Zymomonas mobilis

Two operons encoding xylose assimilation enzymes (xylose isomerase and xylulokinase) and pentose phosphate pathway enzymes (transaldolase, and transketolase) were constructed and introduced into *Z. mobilis* [79]. The transformant, CP4 (pZB5), was able not only to grow but also ferment xylose anaerobically into ethanol. This transformant also acquired the ability to ferment glucose and xylose simultaneously with a slower rate of xylose than glucose utilization. Anaerobic fermentation using a glucose (25 g/l) and xylose (25 g/l) mixture gave an ethanol yield of 25 g/l ethanol with 95% of the theoretical yield within 30 h. The potential of this bacterial strain to ferment poplar hemicellulose hydrolysate to ethanol has been studied [105].

6.2.4
Enhancement of Xylose Fermentation

Xylose fermentation by yeast strains is subject to regulation by the dissolved oxygen and by extraneous materials present in the substrate. It is also affected by the presence of the fermentation end-product, ethanol. Ethanol, at about

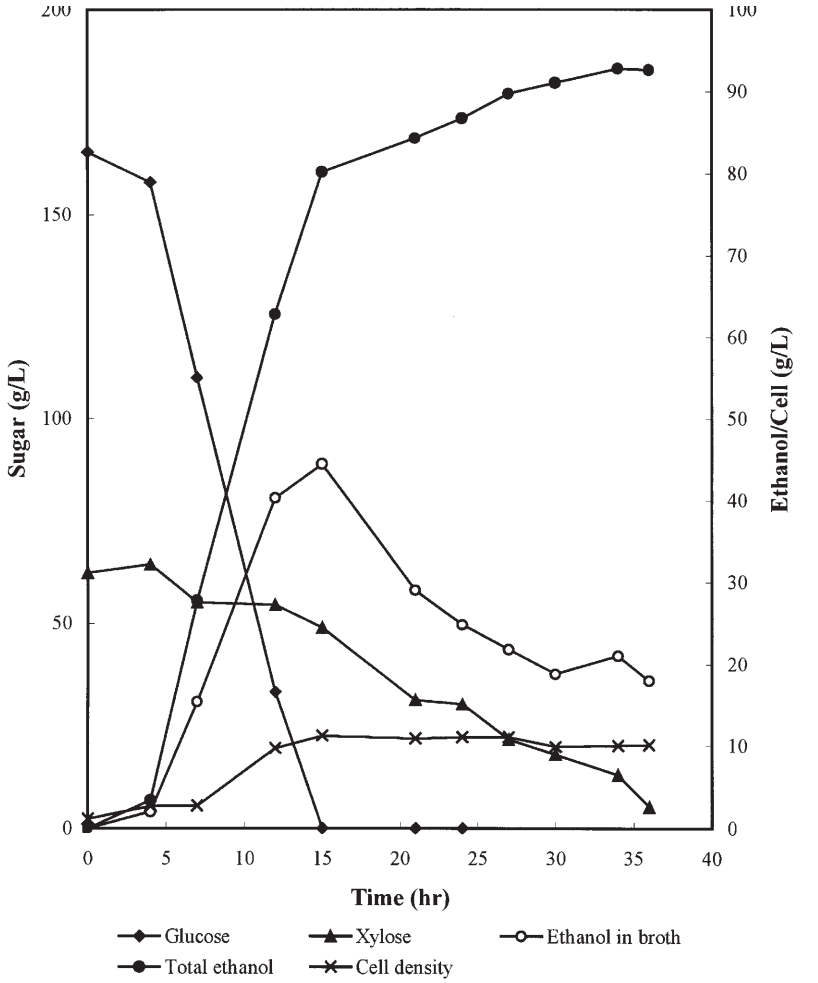

Fig. 10. Fermentation of glucose/xylose mixture with *Saccharomyces* 1400 (pLNH33) in an ALSA fermentor with CO_2 stripping of ethanol [18]

45 g/l, will inhibit the ability of either naturally occurring or genetically modified xylose-fermenting microbes. To overcome the end-product inhibition by ethanol, Tsao et al. [18] used a modified air-lift fermentor (ALSA) coupled with a gas-stripping system to continuously remove ethanol from the fermentation broth. Their results (Fig. 10) show that xylose can be converted into ethanol without the feedback inhibition by ethanol that is encountered under typical stirred-tank fermentation conditions.

7
Municipal and Paper Mill Cellulosic Waste

At least 180 million metric tons of municipal solid waste are generated in the US each year. Over 87% of this material is sent to landfills or incinerators, the rest is recycled for further use. In typical municipal solid waste, about 45% is paper and paper products. Newsprint alone is an estimated 30% of paper-related waste. These cellulosic wastes are the potential substrate for ethanol production [106,107].

Today, about 40% of newsprint is recycled and reused. In 1994 alone, recovered and recycled paper and paper products reached 40 million tons and are expected to reach 60 million by the year 2000 [108]. As more and more paper is being recycled, more and more unusable short fibers are being generated. Approximately 15 to 20% of the fibers are too short to be useful after repulping. The combination of short fibers with fillers, known as recycled paper sludge (RPS), is usually disposed of in landfills as waste paper sludge or subjected to incineration after dewatering [109].

The recycled fibers undergo repeated repulping treatment and the short fibers generated in the waste are chemically swelled and hydrated. This results in a high water-holding capacity of the waste. Because of this high water-holding capacity, the waste is costly to dispose of. The cellulose component of RPS is considered to be more susceptible to hydrolysis by cellulases. To utilize the waste fibers and to reduce the cost of disposal, there have been some efforts to use the RPS either as the substrate for producing cellulases [110,111] or for conversion to ethanol [109,112].

Typically, recycled paper sludge is made up of approximately 50% cellulose, 10% hemicellulose and 40% other materials, and has a moisture content of about 60%. For example, with a dry ton of recovered paper for repulping, there is about 0.15 to 0.2 ton of dry short fibers generated. The wet weight of RPS will be up to 0.5 ton including water, short fiber and other solids known as fillers. Because of its high fiber content, RPS is the type of material that can be effectively utilized and converted into ethanol. The ideal method of producing ethanol from RPS is to use the simultaneous saccharification and fermentation (SSF) process.

SSF of RPS with fungal cellulase and a thermotolerant yeast, *Kluyveromyces marxianus,* was used to convert cellulose fibers of RPS samples to ethanol by Lark et al. [112]. The cellulase loading was 8 filter paper units (FPU)/g dry RPS. About 32 and 35 g/l of ethanol were produced from 180 and 190 g/l dry materials, respectively, after 72 h of incubation. This indicates that at least 72% of the cellulose in the RPS was converted into ethanol. During incubation, the thick slurry of RPS was liquefied within 24 h resulting in the reduction of the water-holding capacity of RPS to 30%–35% of the original.

8
Citrus Processing Waste

Production of orange and grapefruit juice generates large amounts of residues. These residues, mainly peel and segment membranes rich in carbohydrate, are potential feedstock for ethanol production. Upon hydrolysis, the residue gene-

rates a mixture of simple sugars that include glucose, fructose, galactose, galactouronic acid and arabinose. The utilization of the citrus-derived processing waste for ethanol production has been investigated [113]. Grohmann et al. [114] utilized Valencia orange peel mixed with small amounts of segment membranes as the substrate for ethanol production by the recombinant *E. coli* strain KO11. The material was hydrolyzed by a mixture of pectinase (12.4 U/g, Pectinex Ultra SP), cellulase (0.4 IFPU, Celluclast 1.5L) and β-glucosidase at 45 °C for 24 h with no pH control (pH changed from 4.3 to 3.3 upon hydrolysis). Prior to fermentation, hydrolysate was neutralized with $CaCO_3$ to pH 5.8 and filtered to remove solid. Ethanol was produced by *E. coli* KO11 at ethanol concentrations ranging from 28–48 g/l after 72 h of fermentation. *E. coli* KO11 converted all the monosugars in the orange peel hydrolysates to ethanol and a small amount of acetic and lactic acids. Acetic acid was co-produced from galacturonic acid by this bacterium by the following reaction [115]:

$$\text{Galacturonic acid} \rightarrow \text{ethanol} + \text{acetic acid} + 2\ CO_2$$

9
Corn Fiber

Corn fiber is a mixture of corn hulls and residual starch left over after the corn wet-milling process. It comprises about 11% of the dry weight of the corn kernel [116]. Typically, about 4.5 lb of corn fiber are obtained from one bushel of corn. It contains about 70% fermentable sugars, of which 20% comes from starch. Therefore, about 3 lb of readily fermentable sugars (hexoses + xylose) derived from corn fiber can be obtained from each bushel of corn. Currently, corn fiber is mixed with other wet-milling by-products (germ cake, corn proteins and steep liquor) and marketed as gluten feed. Nevertheless, corn fiber can be an abundant and cheap feedstock for ethanol production, particularly in view of its central location. Over 100 million bushels of corn are processed in wet-milling processes per year. This means at least 10 million lb of corn fiber are available as cheap and readily available feedstock for conversion to ethanol. If the corn fiber derived carbohydrates are fermented into ethanol, about 10% more ethanol can be produced from the corn crop. This indicates that about 100 million gallons of extra ethanol can be produced from the corn crop [117].

9.1
Composition of Corn Fiber

A typical corn fiber sample contains approximately 20% starch, 14% cellulose, and 35% hemicellulose. The composition is shown in Table 3. Upon hydrolysis, corn fiber yields glucose, xylose and arabinose as the major carbohydrates.

9.2
Preparation of Corn Fiber for Fermentation

Untreated corn fiber is flaky with residual germ, corn grains, and a waxy coating. Native corn fiber is rather resistant to biological degradation. In order to

Table 3. Composition of Corn Fiber [117]

Component	%
Major component	
Crude fiber	14
Starch	19.7
Protein	11
Lignin	7.8
Others	11.6
Carbohydrate[a]	
Glucose	37.2
Xylose	17.6
Arabinose	11.25
Galactose	3.6

[a] Carbohydrates are derived from crude fiber and starch.

utilize corn fiber for ethanol production, a prior treatment is crucial for subsequent conversion. Various pretreatment options are available that can be used to enhance the solubilization and/or hydrolysis of corn fiber. The pretreatment procedures in the available literature include dilute acid, alkaline, or aqueous hot-water treatments. Each of these has distinctive advantages and disadvantages. Methods for converting corn fiber into ethanol have recently been reviewed [117]. The followings are methods that hydrolyze corn fiber and/or convert corn fibers into ethanol:

(1) Osborn and Chen [118] hydrolyzed the starch fraction of corn fiber with glucoamylase at 90 °C followed by dilute sulfuric acid hydrolysis of hemicellulose at 135 °C for 10 min. Based on the report, over 90 % of the theoretical yield of sugars was obtained.
(2) Grohmann and Bothast [119] used dilute acid to treat corn fiber at 100–160 °C before subjecting the material to enzymatic hydrolysis (saccharification) with cellulase and glucoamylase at 45 °C. The saccharification procedure resulted in the conversion of 85 % of corn fiber into simple sugars. The hydrolysate was then subjected to fermentation by *E. coli* KO11. After 72 h of fermentation, about 30 g/l ethanol was obtained. The yield of ethanol was about 62 % based on sugar consumed.
(3) Moniruzzaman et al. [120] utilized the AFEX technique to treat corn fiber in order to enhance the saccharification by enzymes (e.g., amylases, cellulase, hemicellulase and β-glucosidase). Sugars obtained were over 80 % of the theoretical yield.
(4) Krishnan et al. [121] pretreated corn fiber with dilute hydrochloric acid (0.5 wt %) at 120 °C for 45 min to hydrolyze starch and hemicellulose components prior to SSF with *Saccharomyces* 1400 (pLNH33) as the biocatalyst. Over 40 g/l ethanol was produced after 48 h of incubation (Fig. 11).
(5) Moniruzzaman et al. [122] used enzymatic hydrolysis of ammonia fiber exploded corn fiber to obtain hydrolysate that consisted of 47 g/l of mixed sugars. *Saccharomyces* 1400 (pLNH32) was able to produce 21 g/l ethanol from

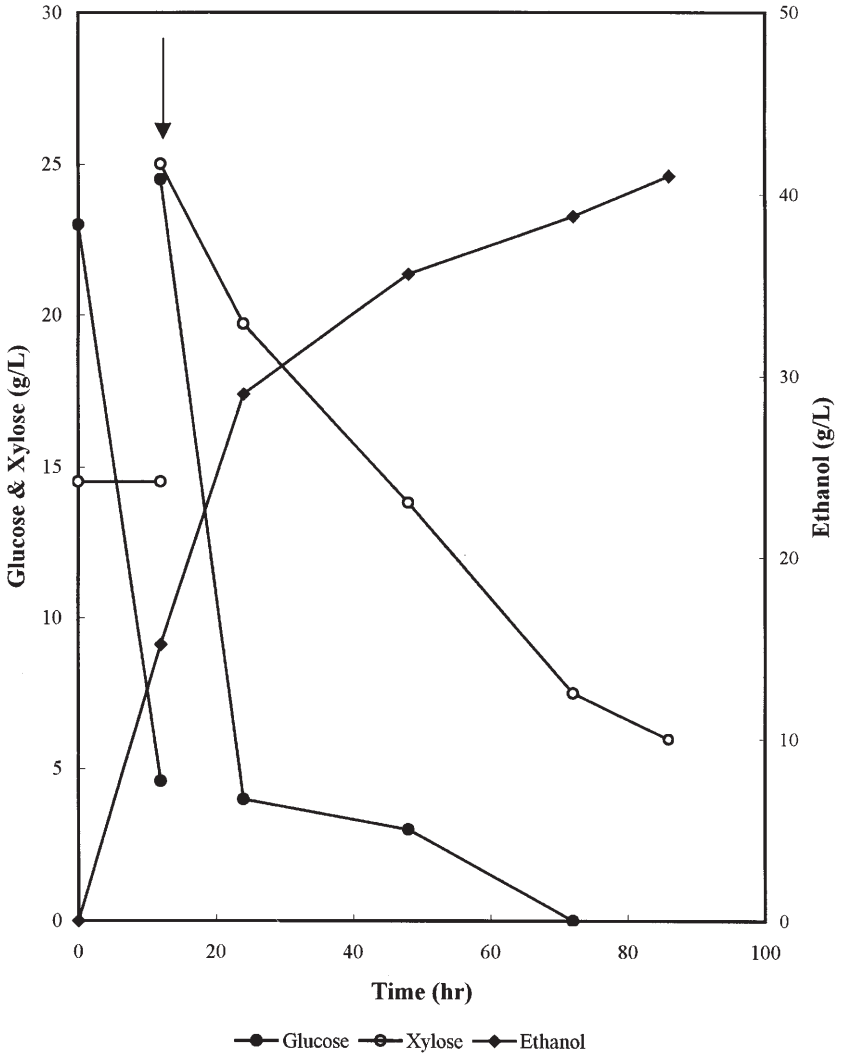

Fig. 11. Single-stage fed-batch SSF process of pretreated corn fiber using *Saccharomyces* 1400 (pLNH33) [121]

hydrolysate with a 98% theoretical ethanol from sugars excluding L-arabinose.

10 Conclusions

As a transportation fuel, ethanol and its derivative, ethyl *tert*-butyl ether (ETBE), have many advantages over gasoline. Ethanol has a lower vapor pressure than gasoline, thereby reducing the smog formation by lowering evapora-

tive emissions. Ethanol also has a high octane value, high heat of vaporization, and other favorable fuel properties. These result in higher efficiency than gasoline if engines are properly optimized. Furthermore, ethanol has a lower toxicity. As a result, even though ethanol has about two-thirds the energy content of gasoline, its benefits as a liquid fuel outweigh its disadvantages.

The current ethanol supply is, in the large part, derived from starch. Nevertheless, vast amounts of agricultural residues and other lignocellulosic biomass can serve as the feedstock for ethanol production. Theoretically, enough ethanol can be produced from cellulosic biomass to meet most of the liquid fuel requirements in the US. The expanded utilization of lignocellulosic biomass for ethanol production can also free starchy crops for food and other uses. In addition, less carbon dioxide emission can be realized if more ethanol can be produced from lignocellulosic biomass and if the market for ethanol as a transportation fuel can be expanded beyond the current level.

For the efficient production of ethanol from lignocellulosic biomass, several procedural obstacles should be overcome. Based on current research progress, the goal of efficient production of ethanol from lignocellulosic biomass can be realized in the near future.

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Production of Multifunctional Organic Acids from Renewable Resources

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Recently, the microbial production of multifunctional organic acids has received interest due to their increased use in the food industry and their potential as raw materials for the manufacture of biodegradable polymers. Certain species of microorganisms produce significant quantities of organic acids in high yields under specific cultivation conditions from biomass-derived carbohydrates. The accumulation of some acids, such as fumaric, malic and succinic acid, are believed to involve CO_2-fixation which gives high yields of products. The application of special fermentation techniques and the methods for downstream processing of products are described. Techniques such as simultaneous fermentation and product recovery and downstream processing are likely to occupy an important role in the reduction of production costs. Finally, some aspects of process design and current industrial production processes are discussed.

Keywords. L-Aspartic acid, Citric acid, Fumaric acid, Itaconic acid, Lactic acid, L-Malic acid, Succinic acid, *Aspergillus niger*, *Aspergillus terreus*, *Lactobacilli*, *Rhizopus arrhizus, Rhizopus oryzae*

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1 Introduction

Many naturally occurring organic acids are multifunctional with the potential to be used as raw materials for the manufacture of biodegradable plastics in additional to their traditional uses in foods and other applications. In order for biologically produced organic acids to be competitive in the market place, the production of these chemicals must be inexpensive. One way to achieve this goal is to utilize inexpensive substrates for organic acid production. Another way is to improve the fermentation process to lower the cost. This paper reviews some recent research developments concerning the aspects of the biological production of some of the wider used organic acids.

2 Lactic Acid

Lactic acid (2-hydroxypropionic acid) is a naturally occurring multifunctional organic acid that is found in many food products, particularly in those which involve natural or processed fermented food preparations. Currently, more than 70% of lactic acid is used as acidulents, food preservatives, and feedstock for the manufacture of calcium stearoyl-2-lactylates in the baking industry. The consumption of lactic acid is estimated to be around 30 million lb in the US with an estimated increase of 6% per year. Therefore, lactic acid is an intermediate-volume specialty chemical used chiefly for food processing.

Recently, lactic acid has been considered to be an essential component for the production of many nonfood products including polylactic acid (PLA). Due to its chemical properties, lactic acid has the potential to become a very large-volume, commodity-chemical intermediate. It can be produced biologically from carbohydrates to serve as the feedstock for slow release carriers, biodegradable polymers, oxygenated chemicals, environmentally friendly solvents, and other intermediates. Recently, lactic acid production has received more attention because of the development of PLA plastics, which are 100% biodegradable [1] and have been approved for use by the Food and Drug Administration. PLA plastics have many characteristics similar to the thermoplastics now used in packaging consumer goods and may become the feedstock for environmentally benign polymers [2].

For the reasons mentioned, lactic acid has the potential to become a very large-volume, commodity-chemical "green" product that can be produced biologically from carbohydrates to serve as the feedstock for polylactate. This potential demand is estimated at 5.5 to 5.7 billion lb/year (or 2.5 to 3.4 million tons). Due to this potential, many large corporations have been involved in product and process development of lactic acid and polylactate production (*Chemical Market Reporter*, October 28 1996).

Lactic acid is produced on the industrial scale by chemical and biological means. The most commonly used synthetic method is based on the hydrolysis of lactonitrile derived from acetaldehyde and hydrogen cyanide. On the other hand, biological production that accounts for about 50% of the current total

acid capacity is primarily carried out by bacterial fermentation of simple sugars. Lactic acid is the smallest molecule that exists in two isomeric forms that also occurs in nature. Chemical methods can only produce a racemic mixture of the stereoisomers. In contrast, biologically produced lactic acid can be obtained in either form of the isomer or as a mixture of the two in different proportions [3].

2.1 Bacterial Fermentation

2.1.1 *Fermentation Pathway*

Lactic acid is a metabolic product of simple carbohydrates produced by many species of bacteria, yeasts, and mycelial fungi mainly through the fermentative metabolic pathway. The stoichiometry for homofermentative production of lactic acid from hexose can be expressed as:

$$1\ C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3$$

Therefore, this bioconversion does not lose any atoms of carbohydrate or produce any carbon dioxide. For the conversion of pentoses, the stoichiometry can be expressed as:

$$C_5H_{12}O_5 \rightarrow C_3H_5O_3 + C_2H_4O_2$$

The bioconversion products are one lactic acid and one acetic acid. Again, there is no loss of material from pentoses [4]. Therefore, lactic acid fermentation has an advantage over other bioconversion processes due to its high product yield and environmental friendliness.

2.1.2 *Industrial Fermentation*

Lactic acid was the first organic acid to be manufactured industrially by fermentation and *L. delbrueckii* is the preferred organism. It facilitates the homolactic fermentation with a temperature optimum of 50 °C and pH of 5.5 – 6.5. Substrates may be simple sugars or starch. Starch is commonly hydrolyzed either by acid or amylases prior to fermentation. Typically, the medium is buffered with excess calcium carbonate and is kept suspended by agitation. The incubation temperature is maintained at about 50 °C until the sugar is metabolized, usually in 48 to 96 h. Carbon dioxide is evolved from added $CaCO_3$ during the lactic acid production stage. This helps to maintain the fermentation under the anaerobic conditions that are required for optimal productivity. Concentrated sweet whey can also be used as the substrate with lactose fermenting *L. bulgaricus* as the biocatalyst due to its ability to ferment lactose [5,6]. Other less expensive unconventional substrates such as food processing wastes and cellulosic materials have also been tested for lactic acid production.

2.1.3 *Process Consideration*

Biological production of lactic acid is complicated primarily due to economical considerations arising from product inhibition and the required downstream processing of dilute aqueous product streams. The standard method of biological lactic acid production is the anaerobic fermentation by *Lactobacillus* in a batch reactor [7]. The conventional process requires the base to be added to the reactor to control the pH and the use of calcium carbonate to precipitate the lactate. This process produces a lactate salt that must be acidified (usually by sulfuric acid) to recover the lactic acid, with calcium sulfate as an undesirable by-product.

2.1.4 *Process Improvement*

To increase the volumetric productivity and to reduce the costs in lactic acid fermentation, high cell density fermentation has been studied through the growth of bacterial cells on activated charcoal, a cell-recycle reactor [8], or a membrane reactor [9]. Recovery of the final product has been examined using liquid extraction [10] and solid adsorbents. This is done either in a product-stripping side stream or by adding directly to the CSTR reactor. Alternatively, in situ product removal during the fermentation offers the advantage of minimizing process waste streams by eliminating the need for reactor pH control and lactic acid recovery.

Recent examples of process improvement have been reported by Davison and Thomson [11] and Kaufman et al. [12]. They studied the simultaneous fermentation and recovery of lactic acid in a biparticle fluidized-bed reactor using *L. delbreuckii* as the biocatalyst. The immobilized bacterial cells (on calcium alginate beads of 0.7–0.8 mm diameter) were fluidized in the liquid media in a column reactor (see Fig. 1). During fermentation, solid particles of lactic acid adsorbent (polyvinylpyridine resin) are added batchwise to the top of the reactor, and fall countercurrently through the biocatalyst. After the adsorbents have fallen through the reactor, they are recovered and the adsorbed lactic acid is recovered. The adsorbents not only remove acid produced but also effectively maintain the broth pH at optimal levels. The increase in lactic acid production is significant. The reported volumetric productivity of 4.6 g/l/h was a 12-fold increase over the reactor without the adsorbents.

2.1.5 *Selection of Adsorbents*

The criteria for selecting the proper adsorbent are: capacity, specificity, ease of regeneration, and the ability to withstand repetitive regeneration. Although a resin exhibiting all of the desired properties has yet to be found, one specific resin, polyvinylpyridine (PVP) Reillex 425, appears satisfactory and has been tested for the recovery of lactic acid [13].

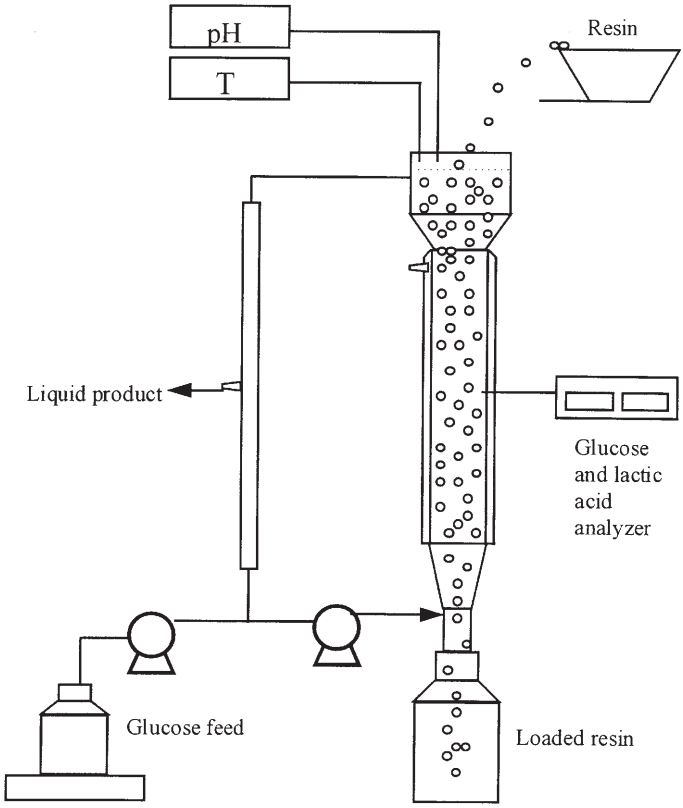

Fig. 1. Schematic diagram of the biparticle fluidized-bed reactor [12]

2.1.6
Product Recovery

Several methods have been explored for the economical recovery of lactic acid from fermentation broth including extraction with solvent, electrodialysis, ion-exchange adsorption (see [14] for review), and reverse osmosis [15]. Wang et al. [16] studied nondispersive extraction for the recovery of lactic acid from broth using a hydrophobic membrane. This method performed favorably when compared to the ion-exchange or the electrodialysis method.

2.1.7
Fermentation of Starchy Materials

The conventional method of lactic acid production from starchy materials such as barley, corn, potato or rice requires pretreatment by gelatinization and liquefaction. This is usually carried out at an elevated temperature of about 90 – 130 °C for at least 15 min, followed by saccharification of the starch by amy-

lases to glucose and subsequent conversion of glucose to lactic acid by fermentation. However, this method involves many reaction steps that require additional reactors. Alternatively, fermentation can be conducted simultaneously with the presence of amylases and biocatalyst known as "simultaneous saccharification and fermentation (SSF)". This method eliminates the need for complete hydrolysis of starch to glucose prior to fermentation. In SSF, the liquefied starch is used in the presence of glucoamylase to continuously hydrolyze starch to produce lactic acid. Linko and Javanainen [17] demonstrated the advantages of SSF by carrying out barley starch fermentation with *L. casei*. Lactic acid concentration as high as 127 g/l was obtained from barley starch within 48 h. The reported lactic acid yield was 98% from an initial starch concentration of 130 g/l. The highest lactic acid accumulation in this report was 162 g/l under high substrate concentration.

2.1.8 *Direct Fermentation of Starchy Materials*

Some starch degrading *Lactobacilli* such as *L. amylophilus* [18] and *L. amylovorus* [19, 20] are able to produce lactic acid from liquefied yet unhydrolyzed cassava, potato and corn starch. However, the final lactic acid concentration is low (< 20 g/l) with low acid yield (< 70%).

2.1.9 *Municipal Solid Waste (MSW)*

L. pentosus was used by McCaskey et al. [21] for the production of lactic acid under static conditions from acid-hydrolyzed MSW. The substrate had the following composition: glucose, 16.4 g/l; xylose, 6.5 g/l; mannose, 14 g/l; and galactose, 4.4 g/l. After 3 d at 32 °C in the presence of $CaCO_3$, 65 g/l lactic acid were produced from 100 g/l total carbohydrate with acid yields ranging from 70 to 85% based on the total sugars consumed. In a subsequent study [22], lactic acid concentration was increased to 78 g/l with 91% lactic acid weight yield when the nitrogen source and phosphate concentration were optimized.

2.1.10 *Cellulosic Biomass*

Cellulose powder and milled newspaper were used by Abe and Takagi [23] as the substrate for lactic acid production by *L. delbruckii* in the presence of cellulase derived from *Trichoderma reesei*. The highest conversion rate was at pH 5. After 120 h of fermentation, the amounts of lactic acid produced from cellulose (100 g/l) and newspaper (50 g/l) were 52 and 23 g/l, respectively.

Chen and Lee [24] studied lactic acid production from dilute acid pretreated α-cellulose and switchgrass by *L. delbruekii* NRRL-B445 in the presence of a fungal cellulase in a fermentor extractor employing a microporous hollow fiber membrane (MHF). This reactor system was operated in a fed-batch mode with continuous removal of lactic acid by in situ extraction. A tertiary amine (alamine

336) was used as the extractant for lactic acid. The extraction capacity was greatly enhanced by the addition of a long-chain alcohol. A solvent mixture of 20% alamine 336, 40% oleyl alcohol, and 40% kerosene was the most effective at pH 5.

2.2 L-Lactic Acid Production by *Rhizopus*

In addition to lactic acid producing bacteria, a few mycelial molds belonging to *Rhizopus* are good lactic acid producers. The ability of *Rhizopus* to produce only L-(+)-lactic acid aerobically under nitrogen-limited environments has been studied [25–28]. Compared to bacterial fermentation, *Rhizopus* requires only inorganic salts. In addition, *Rhizopus* cultures are more tolerant to a low pH environment. Consequently, pH maintenance is not as stringent as bacterial culture during lactic acid fermentation. Furthermore, *Rhizopus* molds are amylolytic that can produce lactic acid from starchy materials directly. For example, *R. oryzae* NRRL 395 was used to ferment starch derived from barley, cassava, corn, oat, and rice to L-lactic acid [25].

Previous work on *Rhizopus* fermentation was carried out using free cells in stirred-tank fermentors. Difficulties were encountered in poor oxygen supply and low fermentation efficiency because of the increase in the fermentation broth viscosity due to the formation of large and soft mycelial aggregates. Therefore, a good oxygen transfer rate and good mixing of *Rhizopus* mycelia in the fermentor are two important considerations for lactic acid accumulation.

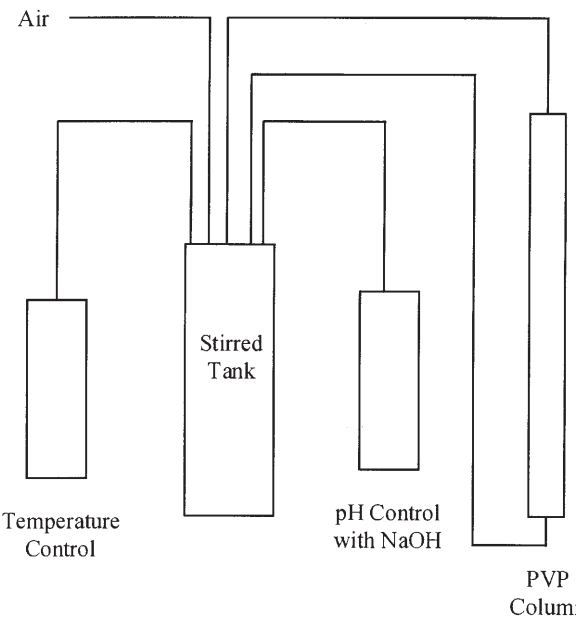

Fig. 2. Schematic diagram of the reactor setup for the simultaneous production and recovery of L-lactic acid [26]

Yang et al. [26] used xylose as the carbon source for *Rhizopus* to grow into mycelial pellets (about 1 mm in diameter). With these restricted mycelial pellets, lactic acid was produced readily and the pellets could be used repeatedly over a 22-d period with good acid yield. When PVP was incorporated into the fermentation system, lactic acid was also produced in the absence of the neutralizing agent. With this fermentor/adsorber system, fermentation can be performed as effectively as when $CaCO_3$ is used (see Fig. 2 for schematic diagram of the fermentation system).

Du et al. [29] studied lactic acid fermentation by *R. oryzae* ATCC 52311 in a bubble column to enhance gas and mass transfers. This column fermentor (3.5-l working capacity) was constructed with a polycarbonate pipe and equipped with a perforated plate located at the bottom of the column to serve as the air sparger. The reactor does not require an agitator and is able to distribute air bubbles evenly. With this reactor, *Rhizopus* produces a higher yield of acid than in a stirred-tank fermentor. To initiate fermentation, the germinated spores are prepared and inoculated into the bubble column. The physical forms (mycelial filamentous or mycelial pellets) of fungal growth can be manipulated by adjusting the pH of the fermentation broth. A final lactic acid concentration of 62 g/l was obtained from 78 g/l glucose (ca. 0.8 g lactic acid/g glucose consumed) with an average specific productivity of 1.46 g/h/g.

2.2.1 *Lactic Acid from Xylose*

R. oryzae NRRL 395 is able to utilize xylose to produce lactic acid, but the production rate is much slower than with glucose as the substrate. Figure 3 shows the kinetics of lactic acid production from glucose and xylose as reported by Yang et al. [26].

2.2.2 *Production of Lactic Acid with Immobilized Cells*

L-Lactic acid fermentation using *R. oryzae* has also been studied using immobilized cells. Dong et al. [30] employed polyurethane foam cubes (macropores larger than hundreds of microns with the pore volume fraction greater than 0.9) as the carriers for *Rhizopus* immobilization. The immobilized cells can reach 450 g fresh cell (67 g dry)/l cube. The amount of immobilized cells was 30% higher than those immobilized with calcium alginate as the carrier [31]. With this immobilized cell system, lactic acid production reached 37 g/l after 8 h with acid yields approaching 78% based on glucose consumed. Furthermore, the immobilized cells can be used in a repetitive manner for more than 10 batches without lose of activity. Likewise, Tamada et al. [32] immobilized *Rhizopus* cells in a polymer prepared from the γ-ray-induced polymer of polyethylene glycol dimetharylate. The specific rates of lactic acid production with the immobilized cells were 1.8 times greater than free cells. Lin et al. [33] immobilized *Rhizopus* mycelium on the plastic surface of a rotary biofilm reactor (RBC) to carry out lactic acid production from glucose.

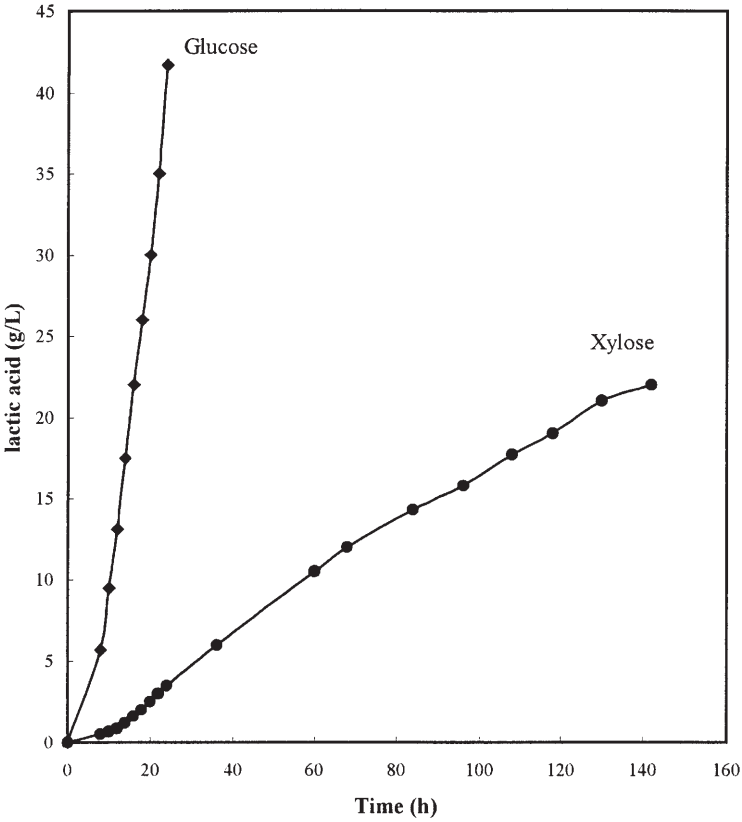

Fig. 3. Kinetics of L-lactic acid production from glucose and xylose by *R. oryzae* NRRL 395 [26]

2.2.3 *Solid-State Fermentation*

Soccol et al. [34] used *R. oryzae* NRRL 395 to produce lactic acid from glucose-impregnated sugar cane bagasse. In the presence of $CaCO_3$, 137 g/l L-lactic acid was produced from 180 g/l glucose with a productivity of 1.43 g/l/h.

3 Citric Acid

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is one of the most widely used naturally occurring organic acids with an estimated worldwide production of over 400,000 tons per year. It is used mostly as an ingredient in food, confectionery, and beverages. Citric acid can form a wide range of metallic salts that are useful as sequestering agents in industrial processes, as anticoagulant blood preservatives, and as antioxidants. Other potential uses include applica-

tion in the cosmetic industry and environmental remediation in which citric acid can be used as a scrubber to remove sulfur dioxide from flue gases. It then reacts with H_2S to give elemental sulfur and the regenerated citrate.

Citric acid can be produced in high productivity and high yield by fermentation of simple sugars mainly by the mycelial fungus, *Aspergillus niger*, although some processes use the yeast *Yarrowia lipolytica* (*Candida lipolytica* or *Saccharomycopsis lipolytica*) with *n*-alkane as the substrate.

3.1 Biochemical Pathways

Because of its economic significance, a great deal of work has been done on the biochemistry of citric acid production. There are four major metabolic sequences involved in citric acid accumulation by *A. niger*: the conversion of carbohydrates via the glycolytic pathway to yield pyruvate; the anaplerotic formation of oxaloacetate from pyruvate; the accumulation of citric acid within the cell membrane; and the secretion of citric acid to outside cells. Step 2 has been shown to be of great importance in the metabolic control of the entire citric acid biosynthesis process. Figure 4 shows the generalized scheme of carbon flow to citrate in *A. niger* [35]. There is considerable information available on the properties of the enzymes involved and the steady state concentration of metabolites during citric acid accumulation. In spite of this, many questions still remain unsettled as to the exact mechanism determining the high yield and high rate of citric acid accumulation. The biochemistry and physiology of citric acid production by *A. niger* and, to a lesser extent, by the yeast *Yarrowia lipolytica* mainly with *n*-alkane as the substrate has been reviewed [35–38].

3.2 Factors Affecting Citric Acid Production

The kinetics of citric acid fermentation by *A. niger* from a sucrose/mineral salt medium has been detailed in a review by Berry et al. [36]. Five successive phases are involved in citric acid accumulation (see Fig. 5):

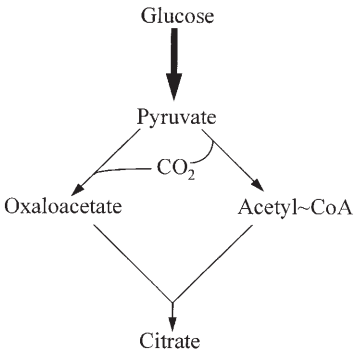

Fig. 4. Generalized scheme of carbon flow from glucose to citrate in *A. niger* [35]

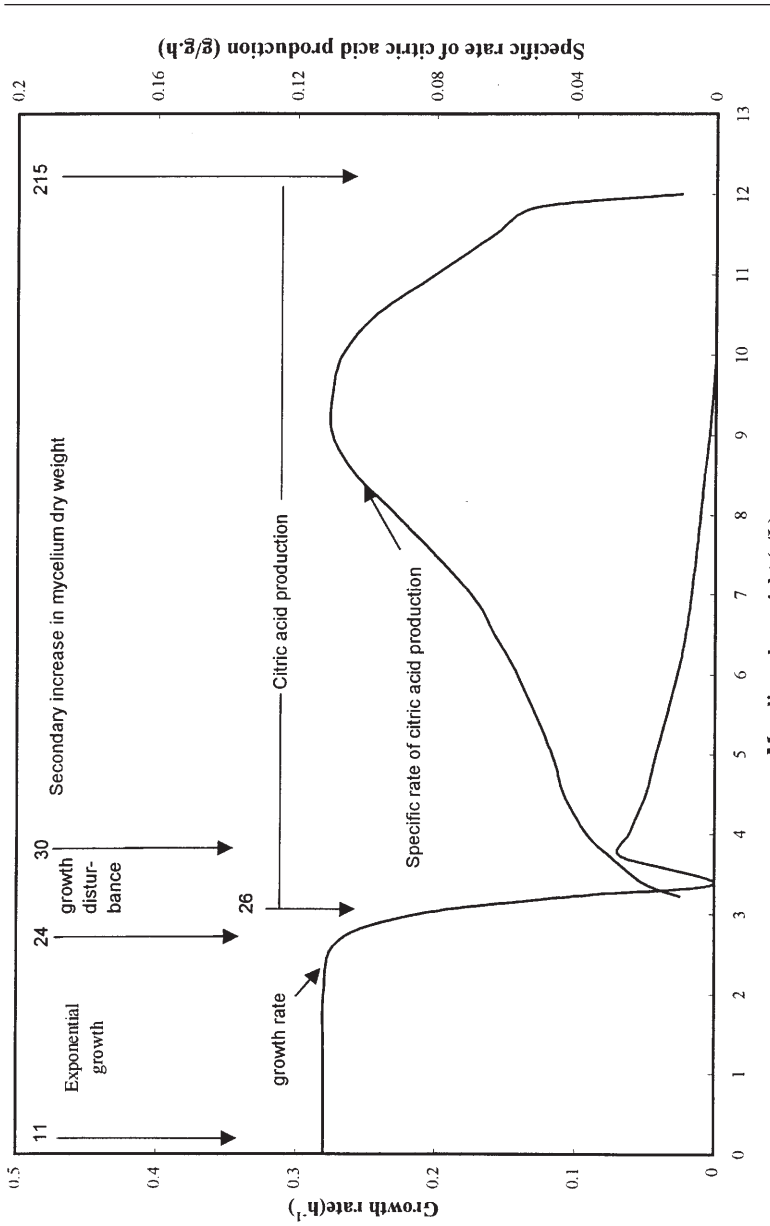

Fig. 5. Relationship between the biomass proliferation, the specific growth rate, and the specific rate of citric acid production [36]

(1) Initiation of spore germination followed by the exponential growth phase,
(2) Period of growth disturbance caused by the exhaustion of nitrogen (e.g. ammonia) and phosphate in the medium,
(3) Citric acid production phase,
(4) Citric acid accumulation phase, and
(5) Declining phase.

3.2.1
Morphology of A. niger

Similar to most mycelial fungi, *A. niger* can grow into a wide variety of physical forms depending on the strain, the initial cell density, the nutrient conditions, the growth environment, and the type of fermentor. For efficient production of citric acid in a stirred-tank fermentor, it is generally agreed that the physical form of mycelial growth is important. The nutritional factors affecting mycelial growth and physical appearance have been studied extensively (see review articles by Mattey [38]).

Nutritional factors affecting the morphology of the developing mycelia greatly influence the performance of the biocatalyst. The development and the morphology of the germinated spores, in turn, determine the high yield and high productivity of citric acid. Many reports indicate that the shape of hyphae growth and their aggregation are crucial to the ultimate yield. Ideally, the hyphae of the newly germinated spores should be abnormally short, bulbous, and should have many short branches [39–42]. It is known that this state of morphological development is brought about by the concentration of Mn^{2+} [39]. Further development of the germinated spores should also form small more or less spherically shaped pellets with a diameter of less than 0.5 mm with a smooth, hard surface [42]. Such pellets are formed when a number of factors are controlled. These are pH, Mn^{2+}, Fe^{2+}, aeration, agitation, and the amount of initial spore inoculum. Based on the experience in the authors' laboratory, developments of this type of morphology and the maintenance of this type of morphology are probably the requisites for successful submerged citric acid accumulation [43].

3.2.2
Effect of Nitrogen

It is generally concluded that nitrogen limitation is required for citric acid accumulation. Normally in defined media, nitrogen is supplied as ammonium sulfate or nitrate. Within 24 h after the inoculation of spores, ammonium ions are rapidly consumed with the excretion of stoichiometric amounts of protons. As a result, the vegetative growth of germinated spores slows down due to the decrease in pH [44] and citric acid accumulation ensues. The optimum nitrogen concentration has been determined to be between 1 and 4 g/l depending on the nature of the substrate, the fungal strain, and the method of fermentation. For an extended period of fed-batch fermentation, the addition of an extra nitrogen source is beneficial to maintain the activity of the biocatalyst [43].

3.2.3
Effect of Phosphate

The effect of phosphate levels on citric acid fermentation is somewhat uncertain. Kubicek and Rohr [45] have shown that citric acid accumulation occurrs when phosphate is limited. The effect of phosphate on balanced growth during citric acid fermentation was studied by Shu and Johnson [46]. Phosphate may be related to the regulation of enzyme systems and the overall energetics. More recent research, however, indicates that the phosphate effect is not very pronounced if the balance of trace elements is maintained [43]. The optimum phosphate concentration was determined to be between 1 and 4 g/l.

3.2.4
Effect of pH

The pH of the medium is vital for a good yield of citric acid. Ideally, the pH should fall below 2 within a few hours after the initiation of the spore inoculation. At high pH, *A. niger* tends to accumulate gluconic acid. This is due to the activation of mycelial-bound glucose oxidase at high pH while at low pH (< 2), this enzyme is inactive [47]. The pH of the medium will also affect the ionization of citric acid. At pH values of about 2, most of the citrate will be present as either citrate^{0} or citrate^{1-}, whereas at an internal pH of about 7, the citrate will be present mainly as citrate^{2-}. It has been suggested that only citrate^{2-} ions can be transported out of the mycelium easily [48].

Therefore, the optimal pH for citric acid accumulation is between 2 and 3. In this pH range, mycelial vegetative growth is not excessive. It also minimizes the formation of other acids such as gluconic and oxalic acid. The optimal starting pH is usually at about 2 – 2.5 but varies depending on the nature of the substrate.

3.2.5
Temperature

Temperatures between 28 – 30 °C are the normal range for obtaining high rates of accumulation and high yields of citric acid. At higher temperatures the fermentation rates are very rapid and abundant mycelial growth occurs causing a low yield of acid with higher levels of by-products. Conversely, at lower temperatures, acid yields are higher with the reduced rates of fermentation. Ideally, the temperature should be high during the initial stage of fermentation and low during the latter stage of acid accumulation.

3.2.6
Aeration

Citric acid fermentation is an aerobic process; an increase in the oxygen supply results in an increase in citric acid yields during submerged fermentation. An interruption in the aeration, even briefly, during fermentation has been known

to have an irreversible impact on the ability of the mycelial pellets to produce citric acid even though the growth and the viability of the biocatalyst remains unaffected. The influence of dissolved oxygen on citric acid accumulation has been examined [49].

3.2.7
Trace Elements

Trace elements are considered to be the main factor influencing the success of submerged citric acid production. They affect the biocatalyst in a two-stage response: inhibit growth at a suboptimal level and stimulate growth at a supra-optimal level. It is well known that some trace elements are more important than others in regulating the proper development of the germinating spores and the subsequent hyphae development. In general, the concentration of trace elements that promote growth is detrimental to acid accumulation. Many variations in the requirements of trace elements for optimal citric acid fermentation have been reported in the literature and have been reviewed [35–38]. The optimum concentration of trace elements varies depending on the substrate, the fungal strain and the mode of fermentation.

3.2.7.1
$Fe^{2+}/Zn^{2+}/Cu^{2+}$

A small quantity of Fe^{2+} accompanied by a limited quantity of Zn^{2+} is essential for obtaining a high citric acid yield. At low Zn^{2+} concentration (below 1 uM) growth becomes limited and citric acid production ensues. If additional Zn^{2+} is added during the production phase (up to 2 uM) then growth can be resumed. Cu^{2+} has been used as an antagonist to Fe^{2+}. Too much Cu^{2+} can affect the uptake of Mn^{2+}.

3.2.7.2
Mn^{2+}

Mn^{2+} has a special effect on the morphological development of the germinated spores, the subsequent mycelial growth, and citric acid production. According to Clark et al. [40], as little as 1 ppb of Mn^{2+} addition will cause a reduction in the citric acid yield by as much as 10%. The detrimental effect of Mn^{2+} on the continuous accumulation of citric acid has been demonstrated by Kubicek et al. [49]. Figure 6 shows the effect of Mn^{2+} addition during citric acid fermentation. A similar effect was also observed with Fe^{2+} addition.

3.3
Industrial Processes

The citric acid production process can be divided into five phases: the preparation of substrates; the preparation of inoculum; the preparation of medium nutrients; the fermentation parameter control; and the product recovery.

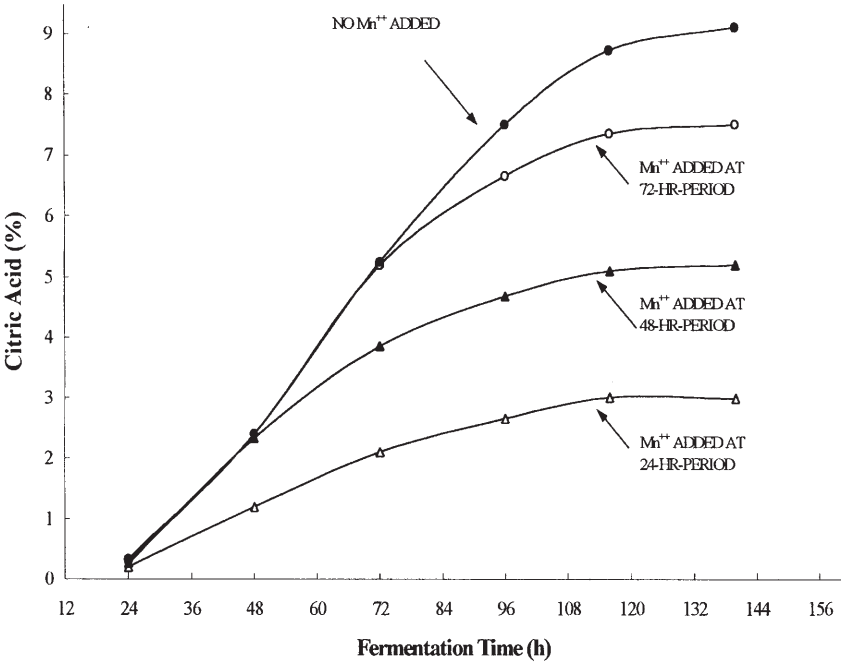

Fig. 6. Effect of addition of 1 ppm of manganese on citric acid production [40]

Three processes are currently in practice for the commercial production of citric acid: the Koji, the shallow-pane, and the submerged process. The Koji process is utilized when a solid substrate is the feedstock. The shallow-pan process, that was used during the early stages of industrial production of citric acid, has more or less been replaced by submerged fermentation due to higher productivity and labor saving.

3.3.1 *Submerged Fermentation*

The submerged process has become the method of choice because it requires less labor to operate, uses less space, is easier to automate, gives a higher production rate, and generates a higher product yield. Several reactor designs such as traditional stirred-tank, bubble fermentor, air-lift fermentor, or air-lift loop fermentor have been used but stirred tanks are the most commonly practiced reactors. Fermentors of all types must be constructed of high-grade stainless steel. This is to avoid the contamination from trace elements, particularly Mn^{2+}, that can be released into the medium under the low pH environment and by the continuous accumulation of citric acid.

Aeration is a significant cost factor in the industrial production of citric acid. The industrial practice uses relatively low aeration rates, initially at 0.01 vvm and rises to 0.5 to 1 vvm as fermentation proceeds. For the bubble column,

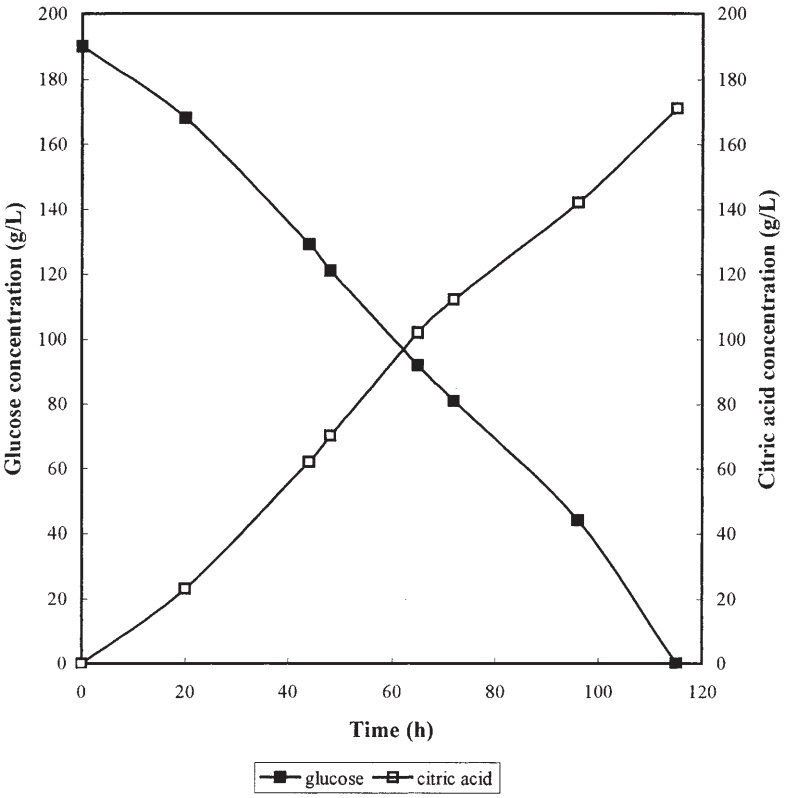

Fig. 7. Kinetics of citric acid production from glucose by *A. niger* in a bubble column [43]. Bubble column: 500-ml working volume; temperature: 35 °C; aeration: 1.5 vvm

enough air has to be supplied to maintain a high dissolved oxygen level and to maintain a suitable rheology of the broth. The reactor may be held above atmospheric pressure to increase oxygen dissolution.

The submerged process takes 3 to 10 d to complete, depending on the method used. Although very high yields are possible, the productivity is a more important consideration on an industrial basis. It is rare that the process is allowed to continue to the maximum yield. The typical kinetics of citric fermentation in a bubble column is shown in Fig. 7 and the kinetics of high yield (up to 360 g/l) in a fed-batch bubble column fermentation is shown in Fig. 8.

3.3.1.1 Substrate Preparation

Traditional submerged fermentation typically uses cane and beet molasses as the source of carbohydrate for citric acid production. All molasses and other crude carbon sources may need pretreatment to regulate the proper amount of heavy metal ions. More recently, starch hydrolysates (corn syrups) have been

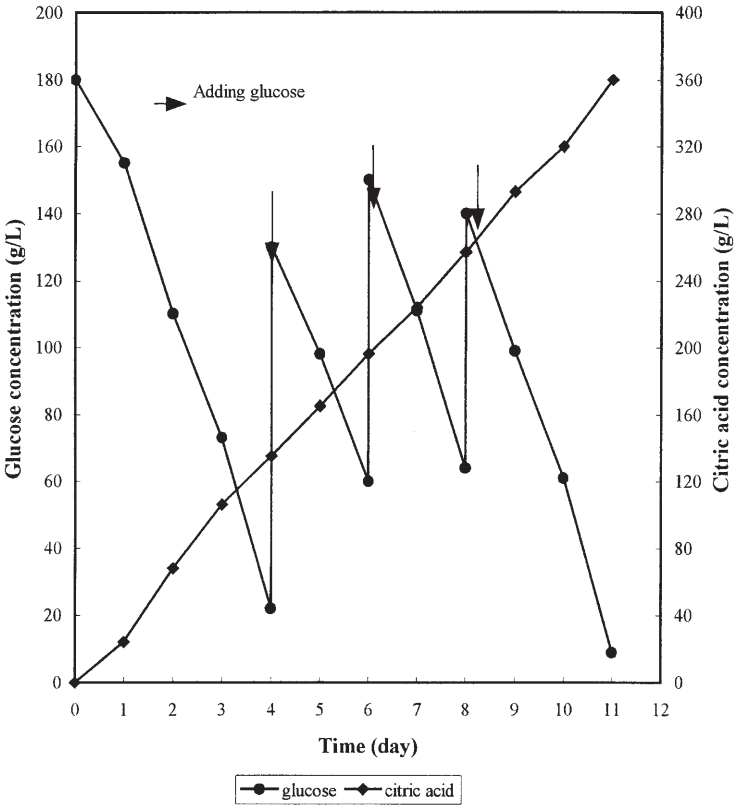

Fig. 8. Fed-batch production of citric acid from glucose by *A. niger* in a bubble column [43]. Bubble column: 500-ml working volume; temperature: 35 °C; aeration: 1.5 vvm

used more frequently than molasses. When crude substrates are to be used it is often necessary to reduce/remove the level of trace elements (e.g., Mn^{2+} and Fe^{2+}). In molasses, the control of metal levels is achieved by treatment with either sodium or potassium ferrocyanide [42] either before or after inoculation of spores. Ferrocyanide not only removes trace metals by chelation but also results in the restriction of fungal growth. A similar effect can be achieved by the addition of excess Cu^{2+}. The amounts of ferrocyanide are in the range of 0.5 to 3 g/l of fermentation medium, normally in the 1.5 to 2 g/l range. Whereas relatively pure substrates such as glucose syrup or sucrose solution are used, ion-exchange resins can be used to remove the trace elements.

3.3.1.2 *Medium Formulation*

Nitrogen is usually supplied as an ammonium salt and/or in combination with other nitrogen sources such as urea. The concentration range varies from 1 to

3 g/l. Phosphates are usually added to give a final concentration of between 1 and 2.5 g/l. Trace elements are supplied according to the original levels in the substrate. Again, careful control of trace element levels is important to limit fungal growth and to achieve maximum citric acid accumulation.

3.3.1.3
Product Recovery

In the final stage of the fermentation, the mycelia are filtered off and citric acid precipitated out of the solution as calcium citrate by the addition of $Ca(OH)_2$. Citric acid is regenerated using sulfuric acid then cleaned up before being finally purified by crystallization. In a typical batch fermentation process, the final acid weight yield is about 80–85% and the recovery efficiency is around 90%. Recently, Annadurai et al. [51] examined the recovery of citric acid from fermentation broth by calcium carbonate precipitation and subsequent acid hydrolysis. They proposed empirical equations to predict the recovery of citric acid by the precipitation method.

3.3.2
The Surface Process

Shallow stainless steel containers are filled with already inoculated medium. Humid air is blown over the surface of the solution for 5 to 6 d, after which dry air is used. As the fermentation progresses, thick mats of mycelium (a few cm in thickness) form on the surface. Citric acid is formed mainly from the bottom layer of cells that have direct contact with the medium. Normally it takes 8 to 10 d to reduce the sugar concentration to 10 to 30 g/l from an original concentration of 200 g/l. The pH of the solution is lowered from the 5–6 range to 1.5–2.0 due to the continuous accumulation of citric acid. The final yield of citric acid is in the range of 80–85% of the sugar utilized [35].

3.3.3
The Koji Process

This method (solid-state fermentation) has been employed for the fermentation of starchy substrates, cellulosic materials, agricultural residues, and food processing wastes. The method utilizes the ability of fungus to produce cellulase and amylases. Since the crude materials normally contain excess trace elements such as Mn^{2+} and Fe^{2+} that interfere with citric acid accumulation, strains that are insensitive to trace elements are often used to carry out this type of fermentation. Typically, solid substrate (pH 5.5) with ca. 70% moisture is inoculated with fungal spores and incubated at 30°C for about 5 to 10 days. With starchy materials, amylases can be supplemented to increase initial sugar concentrations. Likewise, cellulase can be supplemented to enhance cellulose utilization of cellulosic substrates. Often during solid-state fermentation, reactor blockage occurs because of the compacting of solid substrate and fungal growth which causes the channeling of the air supply and temperature and gas

gradients within the reactor. To overcome this mass and heat transfer difficulty, Lu et al. [52] used a multilayer pack-bed reactor to carry out the solid-state fermentation of starchy substrates.

3.4
Production of Citric Acid with Immobilized Cells

In recent years citric acid has been produced on the laboratory scale using *A. niger* immobilized on calcium alginate gel, polyacrylamide gel, polyurethane foam, and disks of rotating biological contactors (RBC). (see [53,54] for related references). For economic reasons, it is impractical to produce citric acid on an industrial scale using the immobilized cell system at the present time.

4
Fumaric Acid

Fumaric acid is a naturally occurring organic acid that is commonly used as a food acidulant and beverage ingredient. With a double bond and two carboxylic groups, fumaric acid is a good source for chemical syntheses. It is useful for making polyesters and other types of synthetic polymers and has many potential industrial applications, such as in the manufacture of synthetic resins, biodegradable polymers and as the intermediate for chemical and biological syntheses. Essentially all of the fumaric acid produced commercially is derived from petroleum-based maleic acid by a catalytic isomerization process.

As the intermediate of the metabolic TCA cycle, fumaric acid is often found as a metabolic product produced by microorganisms. Many species of mycelial fungi produce small amounts of citric, fumaric, malic, succinic, and other organic acids as metabolic by-products during oxidative metabolism. Some mycelial fungi, particularly those belonging to *Rhizopus*, are known to produce significant quantities of fumaric acid from glucose under special cultural conditions [55–57].

Although the production of fumaric acid from either glucose, sucrose, starch, or molasses by fermentation using *Rhizopus* was in commercial operation during the 1940s, it was discontinued due to low productivity and the cheap source of petroleum-derived feedstock.

4.1
Pathway Leading to Fumaric Acid Accumulation

Fumaric acid is an intermediate of the TCA cycle. This oxidative pathway will generate one mole of fumarate per mole of glucose consumed. During active cell growth, however, this pathway cannot lead to a significant accumulation of fumarate. Fumarate generated in the TCA cycle is mainly utilized for the biosynthesis of cell constituents. Early experiments on the production of fumaric acid indicated the possible involvement of the reductive branch of the TCA cycle in fumaric acid accumulation. The carbon dioxide fixing reductive branch is

capable of producing two moles of fumarate per mole of glucose consumed as given in the follow reaction:

$$C_6H_{12}O_6 + 2\ CO_2 \rightarrow 2\ HOOCCH = CHCOOH + 2\ H_2O$$

The enzyme responsible for CO_2-fixation and fumarate accumulation in *Rhizopus* is pyruvate carboxylase (EC 6.4.1.1) which is mainly localized in cytoplasm [58–60]. This enzyme catalyzes the ATP-dependent condensation of pyruvate and CO_2 to form oxaloacetic acid. The ability of *Rhizopus* to incorporate carbon dioxide to produce fumarate has been studied employing $^{13}CO_2$ and $NaH^{13}CO_3$ [61, 62]. Other enzymes involved in fumaric acid accumulation include fumarase and malate dehydrogenase. Both enzymes are also found in cytosol [63]. Kenealy et al. [63] studied the effect of inhibitors (chloramphenicol and cycloheximide) on fumarate accumulation indicating that mitochondria are probably not involved in fumarate accumulation. The accumulation of fumarate from glucose by *Rhizopus* is believed to operate entirely through the cytosolic pathway. The cytosolic pathway is induced in the mycelial fungi under conditions of nitrogen limitation, high carbon to nitrogen ratios, pH of 5.5 or higher and in the presence of $CaCO_3$ [60, 63].

By limiting the nitrogen source, cell growth can be kept to a minimum. During this growth-restricted stage, fumaric acid can be accumulated with a theoretical maximal yield of 2 moles per mole of glucose consumed or 1.29 g fumarate/g glucose consumed on a weight basis [63]. In reality, the cytosolic pathway requires the supply of NADH from the TCA cycle. Therefore, the maximum obtainable yield is about 0.93 g fumarate/g glucose consumed or close to 1.45 moles of fumarate from each mole of glucose [63, 64].

4.2 Nutritional Factors

Rhizopus has very little nutritional demands. Normally, *R. oryzae* and related species require only substrates and inorganic salts to give a satisfactory fermentation performance. The addition of some minor elements may enhance mycelial growth or fumaric acid accumulation. The principal stimulatory metals were found to be Zn^{2+} and Mg^{2+}, which give the maximum stimulation of fumaric acid fermentation at a concentration of 10 and 30 ppm, respectively. Phosphorus, present as the phosphate ion, is required at 200 ppm for optimal production of fumaric acid [64]. Cu^{2+} may inhibit fumarate production at 1 ppm or higher. The effect of Zn^{2+} and other metal ions has been studied [61,64,65]. Similar to citric acid fermentation by *A. niger*, the amount and type of trace elements required to achieve maximum fumaric acid yield vary depending on the fungal strain, the physical state of fermentation, the presence of other trace elements, and the nature of the substrate. Trace metals alone with pH of the growth medium also influence the morphology of mycelial growth. A recent study by Chou et al. [65] shows that very small, distinctive mycelial pellets can be obtained by special medium formulation (see Fig. 9).

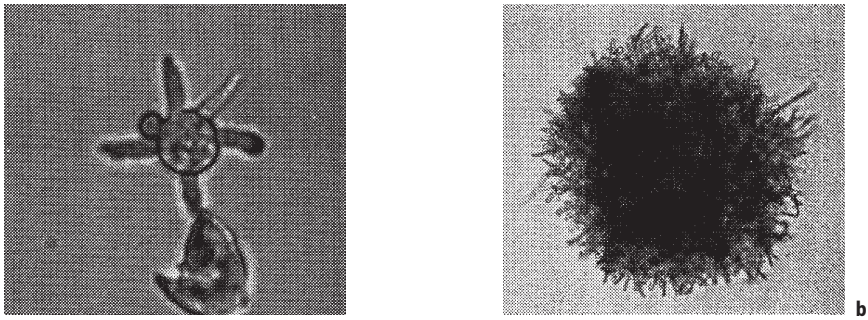

Fig. 9 a, b. **a** Microscope picture of germinated sporangia spores of *Rhizopus oryzae* (courtesy of Y. Chou, Purdue University). **b** Microscope picture of a *Rhizopus* mycelial pellet (courtesy of Y. Chou, Purdue University)

4.3 Submerged Stirred-Tank Fermentation

The production of fumaric acid by fermentation using *Rhizopus* in submerged cultures has been the most commonly employed method. Rhodes et al. [64] studied fumaric acid production by *R. arrhizus* strain NRRL 2582 in a 20-l baffled stainless steel fermentor (10-l working volume) and established the standard for the conditions in stirred-tank fermentation. In their study, the best results were obtained when the continuous addition of $CaCO_3$ as the neutralizing agent was used. Other neutralizing agents such as NaOH and KOH are not as satisfactory due to the effect of the ionic strength from the accumulation of soluble fumarate salts when the concentrations in the broth reach 35–40 g/l. Conditions established by Rhodes et al. to obtain the best yield (0.65 g fumaric acid/g sugar consumed) of fumaric acid include a simple salt solution, 100–160 g/l sugar concentration, 0.5 vvm aeration, 300 rpm agitation rate, 33 °C incubation temperature, and pH of 5.5–6.0. In most cases, fumaric acid produced accounted for about 80 % of the total organic acids accumulated.

To initiate the fermentation, *Rhizopus* spores are inoculated directly into the fermentor. The production of calcium fumarate normally follows a regular pattern. Initially, there is slow utilization of sugars accompanied by the development of mycelial growth and the initiation of acid production. This is followed by the rapid consumption of sugars and the accumulation of fumarate. However, the solubility of calcium fumarate in the broth is limited. During fermentation, growing mycelia form interlocking hyphae often mingled with solid calcium carbonate and calcium fumarate crystals. This results in the formation of a very viscous solution which leads to an increase in diffusion and oxygen transfer restrictions, an operational problem that slows down the fermentation rate.

In fumaric acid fermentation, the most critical control factor is the amount of nitrogen present. Nitrogen is the main factor in the regulation of mold vegetative growth or fumaric acid production. On the other hand, hydromechanical forces influence the morphology of fungi and the bioparticle sizes. Therefore,

minimizing the branch extension of the hyphae to obtain small mycelia particles is important [65].

4.4 Problems Encountered During Fumaric Acid Fermentation

The fumaric acid fermentation by *Rhizopus* is an aerobic process that requires oxygen. Usually, an agitated-sparged stirred-tank fermentor is employed to achieve a good oxygen transfer rate. There are several problems encountered during submerged stirred-tank fermentation. In a typical fumaric acid fermentation, the presence of a neutralizing agent, particularly $CaCO_3$, is required to maintain the proper pH and to supply CO_2 as the co-substrate. The solubility of calcium fumarate and fumaric acid are only 21 and 7 g/l, respectively. Due to its low solubility, fumarate crystallizes easily and forms conglomerates with fungal mycelia that result in complication during fumarate recovery. Gangl et al. [66] used Na_2CO_3 to neutralize the fumaric acid produced in order to avoid the complicated product recovery process. Federici et al. [67] used KOH/K_2CO_3 as the neutralizing agent and CO_2 source for the conversion of starch hydrolysate (glucose syrup) to fumaric acid. In general, fumaric acid productivity (g/l/h) is lower when Na_2CO_3 is the neutralizing agent.

Another difficulty encountered is the tendency of *Rhizopus* sporangiospores to grow into mycelial mats or mycelial clumps. The growing mycelia will anchor onto the inside elements of the reactor such as the bafflers, propellers, and heat exchanger (see Fig. 10, unpublished observation). This results in the inter-

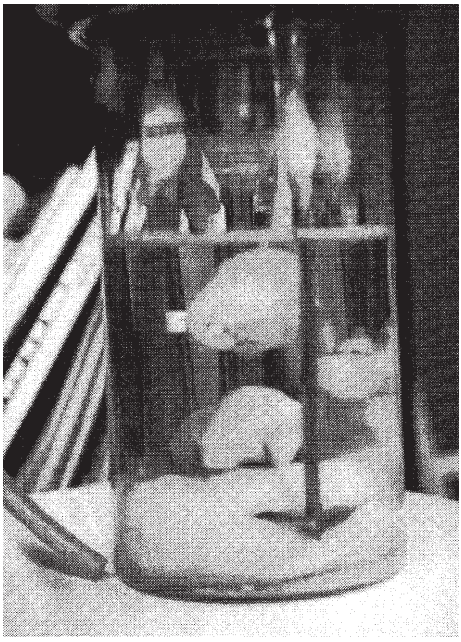

Fig. 10. Picture of *Rhizopus* mycelia attached to a fermentor (unpublished observation)

ference of oxygen and mass transfers and therefore enhanced ethanol production at the expense of acid accumulation. One way to avoid this problem is to grow the cells in a specially formulated growth medium to obtain small compact mycelial pellets (0.5–1 mm, see Fig. 9) before subjecting the cells to the non-growth fermentation medium. Other methods include the use of an air-lift loop fermentor or rotary biofilm contactor (RBC) to carry out the fumaric acid fermentation.

4.5 Air-Lift Loop Fermentation

Du et al. [68] employed a laboratory air-lift loop fermentor for fumaric acid fermentation. This fermentor (10-l working capacity) has a concentric draught tube inside the reactor that is used as the riser section to form an inner gas-liquid loop (see Fig. 11 for configuration). This reactor does not require an agitator. It is also designed to provide an even distribution of air bubbles and carbon dioxide in the broth that is necessary to provide a high yield of acid. With *R. oryzae* ATCC 20344, a fumaric acid production rate of 0.81 g/l/h was obtained with a weight yield of 0.75 g/g glucose consumed. In another study, Du et al. [69] conducted fumaric acid fermentation in an air-lift loop fermentor with two different sizes of mycelial pellets. Results show a high yield of fumaric acid (0.865 g fumaric acid/g glucose consumed) is obtained with smaller pellets.

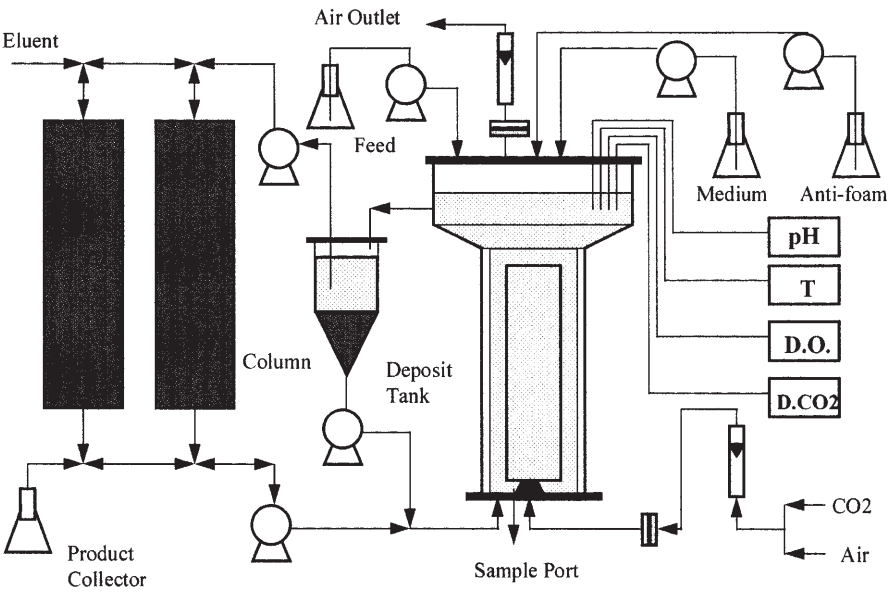

Fig. 11. Air-lift loop fermentor with product recovery system [69]

4.6
Fumaric Acid Production with a Rotary Biofilm Contactor (RBC)

A rotary biofilm contactor (RBC) is a vessel that contains plastic plates that act as the supports for fungal mycelial growth. The plates are mounted onto a horizontal shaft and placed in the vessel containing the medium (see Fig. 12 for reactor configuration). During the growth stage, *Rhizopus* mycelia are grown onto the plastic surface of the plates and form the "biofilm" and are immobilized on the surface of the plates (Fig. 13). During the fermentation stage, the shaft rotates slowly exposing the biofilm periodically to the gas space on the upper portion of the RBC for good oxygen absorption, and to the lower portion with the liquid medium for substrate uptake and excretion of fumaric acid. Similar types of reactors have been used for industrial wastewater treatment [71].

Cao et al. [70] operated a 2-l RBC fermentor with a 0.9-l working liquid volume for fumaric acid production with $CaCO_3$ as the neutralizing agent. With *R. oryzae* ATCC 20344, a fumaric acid production rate of 3.78 g/l/h was obtained with a weight yield of 0.75 g/g glucose consumed. A final calcium fumarate concentration of 75.5 g/l was obtained after 24 h from an initial glucose concentration of 98.7 g/l. The volumetric productivity achieved using the RBC is almost four times higher than that using a stirred-tank system (0.94 g/l/h) with $CaCO_3$ as the neutralizing agent.

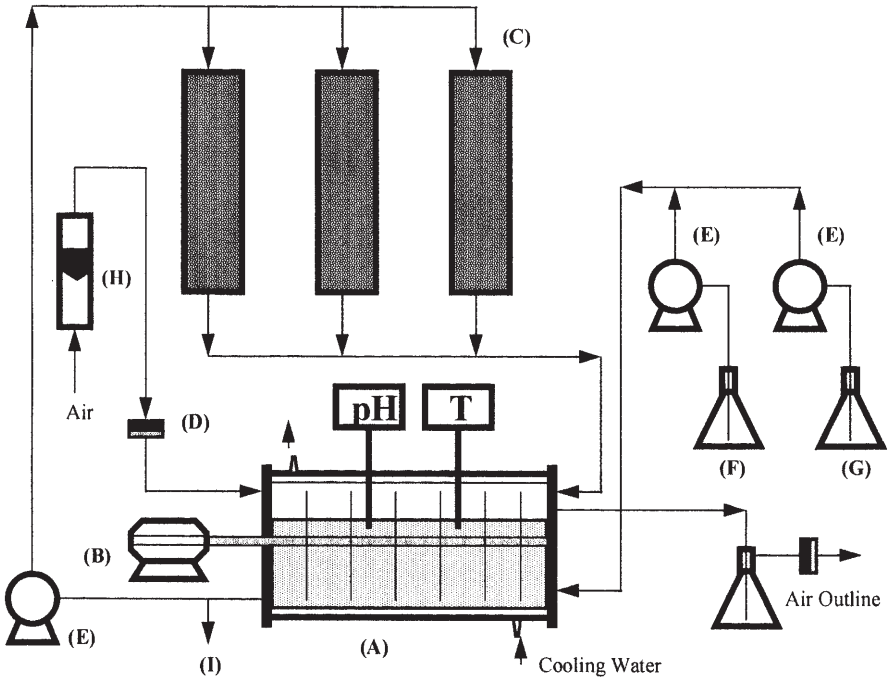

Fig. 12. Rotary biofilm contactor with product recovery system [72]

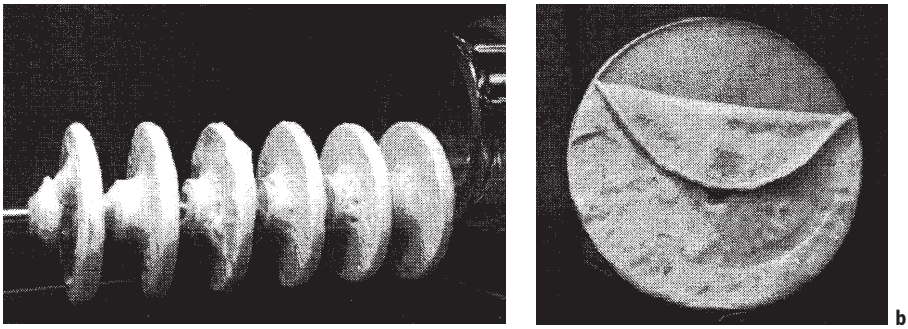

Fig. 13 a, b. **a** *Rhizopus* mycelia immobilized on the surface of plastic plates [70]. **b** Biofilm on solid support [70]

In another experiment, an adsorption column (with anion-exchange resin or polyvinylpyridine) was coupled to the RBC to carry out simultaneous production and recovery of fumaric acid without the addition of $CaCO_3$ (for configuration, see Fig. 12). With this system, Cao et al. [72] were able to obtain a fumaric acid yield of over 90% of the theoretical maximal yield (0.85 g fumaric acid/g glucose consumed). The specific productivity of 4 g/l/h obtained with this system is higher than RBC fermentation with $CaCO_3$ as neutralizing agent. The adsorbed acid can be recovered as pure product after elution with NaCl followed by the acidification of the recovered sodium fumarate. The same pregrown biofilm in the RBC can be used repeatedly provided the immobilized fungal mycelial are activated periodically with nitrogen-rich solution.

4.7 Production of Fumaric Acid from Xylose

Fumaric acid can also be produced from xylose. The rate of xylose fermentation is much slower than with glucose with a specific productivity of only about 0.075 g fumaric acid/h/g biomass. Kautola and Linko [73] used immobilized *R. arrhizus* with polyurethane foam to ferment xylose. A specific productivity of 0.087 g/l/h was obtained when the initial xylose concentration was 100 g/l and the resident time was 10.25 days.

4.8 Production of Fumaric Acid with Immobilized *Rhizopus*

Fumaric acid production using immobilized *Rhizopus* cells has also been studied. Petruccioli et al. [74] immobilized *R. arrhizus* NRRL 1526 on polyurethane sponge to carry out repeated batch fumaric acid production from glucose syrup with KOH/KCO_3 as the neutralization agent and CO_2 source. Although the yield (12.3 g/l) is low, it provides the possibility of using immobilized *Rhizopus* for the continuous production of fumaric acid.

4.9
Fumaric Acid from Starch Hydrolysates

Fumaric acid production from starch hydrolysate by *R. arrhizus* NRRL 1526 was studied by Federici et al. [75] in a 3-l stirred-tank fermentor with $CaCO_3$ and KOH/KCO_3 as the neutralizing agent and CO_2 source. The fermentation conditions for fumaric acid production by this fungus from potato flour has been optimized by Moresi et al. [76].

5
L-Malic Acid

Similar to fumaric acid, L-malic acid is also a naturally occurring four-carbon dicarboxylic acid and an intermediate in the TCA cycle. It has been used in many food products, primarily as an acidulant. L-Malic acid is compatible with all sugars with low hygroscopicity and good solubility. In addition, it has therapeutic value for the treatment of hyperammoemia and liver dysfunction and as a component for amino acid infusion. L-Malic acid has been the subject of interest because of its increased application in the food industry as a citric acid replacement and its potential use as a raw material for the manufacture of biodegradable polymers.

Traditionally, L-malic acid has been obtained from its natural source, apple juice. However, this is impractical due to the small quantities in which it occurs. Large-scale production of malic acid can be achieved by chemical methods through the hydration of either maleic or fumaric acid. It can also be obtained by biological hydration of chemically derived fumaric acid to L-malic acid mediated by fumarase (fumarate hydratase, EC 4.2.1.2) or by the fungal fermentation of simple sugars. The synthetic method generates a racemic mixture of the stereoisomers, D- and L-malic acid. In contrast, the biological method produces only L-malic acid. For human consumption, L-malic acid is preferred. Likewise, the single isomer is preferred as the feedstock for biodegradable polymers. Large-scale production of L-malic acid from simple sugar or cheap cellulosic biomass is possible. However, it is expensive because of the relatively low concentration of acid produced and the high product recovery cost. The most common method for the biological production of L-malic acid is by enzymatic hydration. It is mediated by fumarase of chemically derived fumaric acid using *Brevibacterium* strains [77–79].

5.1
Malate from Fumarate

Fumarase is an enzyme component of the TCA cycle that catalyzes the reversible reaction of fumarate to L-malate with equilibrium favoring malate production. It is a soluble enzyme with high turnover number. In one report, fumarate content in some organisms can be as high as 1000 mg/kg of wet cells [80]. Theoretically, a malate weight yield of 115% can be obtained from fumarate. However, in reality, a weight yield of 90–95% is often obtained.

Since the 1970s, malate has been produced from fumarate using immobilized *Brevibacterium* as the biocatalyst in a continuous process on a large scale [77]. Because fumarase is one of the essential enzymes involved in the basic oxidative metabolism, it is likely that fumarase is active in most living organisms. However, high fumarase activity has been found in only a few bacterial species [81]. Eukaryotic microorganisms such as mycelial molds or yeasts are not known to have very active fumarase activity for in vivo conversion of fumarate to malate. There are few reports using immobilized baker's yeast, *Saccharomyces cerevisiae*, for malate production from fumarate. However, the specific activity of yeast conversion is only about one-third compared with those by bacterial systems [82]. Neufeld et al. [83] studied L-malate formation by immobilized *S. cerevisiae* that was amplified for fumarase in the presence of a surfactant. The highest specific activity achieved was reported to be three times higher than the bacterial system. Likewise, Wang et al. [84] reported that a genetically modified yeast strain, *S. cerevisiae* SHY2, possesses high in vivo fumarase activity.

5.2
Fermentative Production of L-Malic Acid

L-Malic acid can also be produced from simple sugars in significant quantities by a number of *Aspergillus* species [85] and by the wood mushroom, *Schizophyllum commune* [86]. Similar to fumarate fermentation by *Rhizopus*, special cultural conditions are required for the production of L-malic acid by *Aspergilli* from glucose. *A. flavus* produced a high weight yield of L-malic acid from glucose in a stirred-tank fermentor with $CaCO_3$ as the neutralizing agent. The high yield of malic acid is believed to be due to the ability of the fungus to incorporate CO_2 into the product during the L-malic acid producing phase [85]. The activity of pyruvate carboxylase and its location in the cytoplasm of the cells are believed to be the key factors in the high L-malic acid yield.

5.3
Production of L-Malic Acid by *A. flavus*

Battat et al. [87] used *A. flavus* ATCC 13697 as the biocatalyst for the production of malic acid from glucose in a 16-l stirred-tank fermentor. The optimal fermentation conditions are as follows: agitation rate, 350 rpm; Fe^{2+}, 12 mg/l; nitrogen (as ammonium sulfate), 271 mg/l; phosphate, 1.5 mM. Total amount of $CaCO_3$ added was 90 g/l. Fermentation was carried out at 32 °C for up to 200 h. Under the aforementioned conditions, 113 g/l of L-malic acid were produced from 120 g/l glucose utilized with an overall productivity of 0.59 g/l/h. Based on the molar yield, it was 128 % for malic acid and 155 % for total acid (malic, fumaric and succinic acid). The increase in acid accumulation during the course of incubation coincides with the increase in the activities of NAD^+-malate dehydrogenase, fumarase and citrate synthase.

5.4
Production of L-Malic Acid by *Schizophyllum commune*

Kawagoe et al. [88] used *S. commune* as the biocatalyst for the production of malic acid from glucose (50 g/l) in an 8-l air-lift column fermentor (7.2-l working volume). The optimal fermentation conditions are as follows: superficial air velocity, 1.7 cm/s; temperature, 27°C; nitrogen (as ammonium nitrate), 10 g/l; $CaCO_3$, 50 g/l. After 110 h of fermentation, 43 g/l of L-malic acid were produced from 50 g/l glucose.

5.5
Mixed Culture Fermentation

L-Malic acid can also be produced from glucose using a combination of a fumaric acid producer (*Rhizopus arrhizus*) and an organism with a high fumarase activity in the same fermentor [89,90].

5.6
Biochemical Aspects of L-Malic Acid Production

The cytosolic location of pyruvate carboxylase responsible for the accumulation of high concentrations of malic acid has been demonstrated in *A. flavus* [87]. This enzyme and other related enzymes (e.g. malate dehydrogenase) are induced in the mycelial fungi under conditions of nitrogen limitation, high carbon-to-nitrogen ratios, a pH of 5.5 or higher, and in the presence of $CaCO_3$. The protein synthesis inhibitor, cycloheximide, inhibits both malate accumulation and malate dehydrogenase activity without affecting fumarase activity [91]. Similarly, inhibitors of pyruvate carboxylase, such as avidin, inhibit the formation of malic acid. These results suggest that both malate dehydrogenase and pyruvate decarboxylase are required for malic acid accumulation.

Recently, *S. cerevisiae* has been studied in regard to the biochemical regulation of malic acid production [92,93]. This common yeast has been shown to produce small amounts of fumaric and malic acid (less than 10 g/l) from glucose under environmentally stressed conditions. Similar to the malic acid production pathway of *Aspergillus*, the cytosolic reductive pathway of acid synthesis and accumulation has also been shown in *S. cerevisiae*. A ^{13}C NMR study following the label from glucose to malic acid indicates the following reactions lead to malic acid accumulation: pyruvate → oxaloacetate → fumarate → malate. The involvement of cytosolic fumarase in the conversion of fumaric acid to malic acid has been collaborated recently. Wang et al. [94] have shown the ability of a cytoplasmic respiratory deficient mutant of *S cerevisiae* to convert fumarate to malate without the participation of mitochondria fumarase.

6
L-Aspartic Acid

Aspartic acid is an amino diacid that is commonly used as an ingredient in the synthesis of the artificial sweetener, Aspartame. Aspartic acid is also a useful

monomer for the manufacture of polyesters, polyamides, and other chemical derivatives. The polymeric form of aspartic acid, polyaspartic acid, is an ingredient in cleaning compounds that can be used as a replacement for EDTA and other widely used chemicals. The potential market for polyaspartate can be as high as US$ 450 million per year. The key to a wider use of polyaspartate is a cheap source of L-aspartic acid (*Chemical Market Reporter*, 18 October 1996).

L-Aspartic acid can be produced by direct fermentation of sugars using bacterial strains. However, commercially it has been produced by the amination of fumaric acid by immobilized bioctalysts that have high aspartase (EC 4.3.1.1) activity in a fixed-bed reactor. Suitable microbes for the industrial bioconversion of fumaric acid to L-aspartic acid include strains of *Brevibacterium*, *Corynebacterium*, *E. coli*, and *Pseudomonas*. A weight yield of 110% can be obtained in the conversion of fumaric acid to aspartic acid as shown in the following:

$$\text{fumaric acid} + NH_3 \rightarrow \text{L-aspartic acid}$$

The first commercial production of L-aspartic acid was started in 1973 by the Tanaba Seiyaku Company, Japan. The process uses aspartase contained in whole microorganisms and involves the immobilization of *E. coli* on polyacrylamide gel or carrageenan. The immobilized cells are then subjected to treatment in order to increase cell permeability. The substrate, fumaric acid, is dissolved in a 25% ammonia solution and the resulting ammonium fumarate is then passed through the reactor containing the immobilized *E. coli*. The reaction is exothermic and the reactor has to be designed to remove the heat produced. The conversion of fumaric acid to aspartic acid is more economical than the direct fermentation of sugars. The key to economical production of L-aspartic acid for expanded use is a cheaper and more abundant source of fumaric acid.

7 Succinic Acid

Succinic acid is a flavor-enhancing organic acid used in dairy products and fermented beverages. This acid and its derivatives are widely used as specialty chemicals with applications in polymers, foods, pharmaceuticals, and cosmetics. Succinic acid is a valuable four-carbon intermediate that can be converted by catalytic processes into 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone. It can also be easily esterified to dimethyl succinate, which is marketed as an environmentally friendly solvent [95].

Succinic acid is currently manufactured by the hydrogenation of maleic anhydride to succinic anhydride, followed by hydration to succinic acid. A fermentation process for succinic acid production is desirable because in such processes, renewable resources such as starchy crops and other agricultural products can be used as feedstock for the biological production of succinic acid. It addition, a high purity product, which is required as raw material for polymer manufacture, can be obtained.

Succinic acid is one of many organic acids (e.g. acetic, butyric, caproic, formic, propionic and succinic acid) produced from glucose by many rumen anaerobic bacteria. Only a very few accumulate succinate as the anaerobic end-

product in significant concentration. One of such rumen anaerobes, *Fibrobacter succinogenes*, is capable of utilizing cellulose to produce succinic acid. With pulpped shredded office paper, *F. succinogenes* produced 3.2 g/l succinate after 90 h from 10 g/l substrate with acetate (0.58 g/l) and formate (0.07 g/l) as the co-products [96]. By far the best succinate producer is *Anaerobiospirillum succiniciproducens*, a non-ruminal anaerobic bacterium. According to Nghiem et al. [97], a maximum of 32 g/l of succinic acid was produced by this anaerobe in 27 h at pH 6.0 with a CO_2 sparge rate of 0.08 l/l/min with a yield of 0.99 g succinate/g glucose consumed.

The degree of succinic acid accumulation by *A. succiniciproducens* is influenced strongly by the culture pH and by the availability of CO_2. Samuelov et al. [98] studied the effect of CO_2 and pH on growth, fermentation kinetics, carbon balance, and the profile of the product formed by this organism. They also measured the changes in the levels of key catabolic enzymes with respect to pH and available CO_2. They arrived at the following conclusions (see Fig. 14):

(1) Glucose is metabolized via the Embden-Meyerhof-Parnas (EMP) route to generate phosphoenolpyruvate,
(2) Oxaloacetate is formed from PEP with CO_2 as the co-substrate,
(3) Reduction of oxaloacetate results in malate formation,
(4) Under excess CO_2/HCO_3^- growth conditions, phosphoenolpyruvate carboxykinase levels increase while lactate dehydrogenase and alcohol dehydrogenase levels decrease,
(5) The accumulation of succinic acid is a growth-associated event, resulting from the regulation of electron sink metabolism.

In contrast, Millard et al. [99] showed that the over expression of phosphoenolpyruvate carboxylase in *E. coli* is responsible for succinic acid accumulation and not phosphoenolpyruvate carboxykinase.

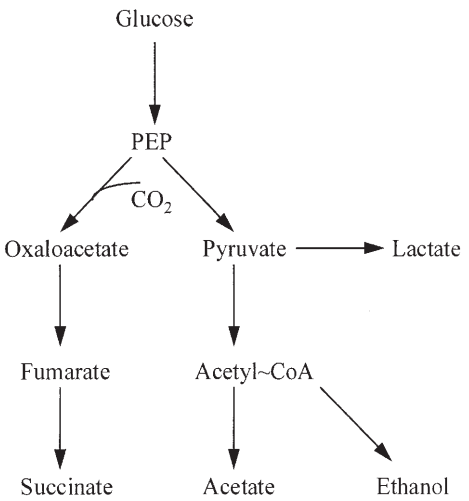

Fig. 14. Proposed catabolic pathway for glucose fermentation in *A. succiniciproducens* [98]

Table 1. Summary of fermentation results obtained at 32 h [104]

Succinic acid concentration (g/l)	47.5
Succinic acid yield (g/g fumaric acid consumed)	93%
Specific productivity of succinic acid (g/g biomass/h)	0.183
Volumetric productivity of succinic acid (g/l/h)	1.48
Succinic acid/acetic acid (g/g)	11.4

Succinic acid can also be generated from fumarate [100] or citrate [101] in the presence of a readily metabolizable carbon source to serve as the hydrogen donor. When citrate is the hydrogen acceptor, it is split into oxaloacetate and acetate by citrate lyase. Oxaloacetate is in turn converted into succinate [102]. The rate of conversion and yield of succinate from fumarate can be enhanced by the amplification of genes that synthesize fumarate reductase [103, 104]. Table 1 shows the fermentation results reported by Wang et al. [104]. In addition, succinic acid can be generated from glucose with mixed culture fermentation, in which fumarate produced by a *Rhizopus* culture is converted into succinate by a bacterial culture [105].

8 Itaconic Acid

Itaconic acid (methylene succinic acid) is an unsaturated five-carbon dicarboxylic acid. It is a by-product of the destructive distillation of citric acid. The reaction leading to itaconic acid from citric acid is as follows:

$$\text{citric acid} - H_2O \rightarrow \text{aconitic acid} - CO_2 \rightarrow \text{itaconic acid} + \text{citraconic acid}$$

Aconitic acid is also present in sugar cane juice and is believed to interfere with sucrose crystallization. Removal of calcium aconitate by sugar refiners, and its subsequent conversion to itaconic acid by heating the solution, was the principal source of itaconic acid until the fermentation process was developed.

Itaconic acid is a useful feedstock for the synthesis of polymers. This is due to the ability of the methylene group of this acid to engage in polymerization reactions. The resulting polymers are used in carpet backing and paper coating. Another significant reaction product of itaconic acid is the formation of N-substituted pyrrolidinones with amines. The products are used in detergents, shampoos, and other products in which their surface activity is useful [106].

In 1929 Kinoshita [107] identified itaconic acid as the major metabolic product of *A. itaconicus*. Later research showed that *A. terreus* is a better biocatalyst for itaconic acid accumulation. A number of yeast strains belonging to *Candida* and *Rhodotorula* [108] can also accumulate a limited amount of itaconic acid. Patents on the industrial production of itaconic acid using *Aspergilli* as the biocatalyst from molasses were issued in 1961. The currently preferred industrial process uses improved strains of *A. terreus* as the biocatalyst. The most often studied itaconic acid producers are *A. terreus* NRRL 265 and *A. terreus* NRRL 1960.

The biochemistry of the itaconic acid biosynthesis is very similar to that of citric acid. The general pathway from hexose to pyruvate (via the EMP pathway)

to the TCA intermediate is the same. The metabolic flux limitation appears to occur at the same place as in the citric acid biosynthesis [109], although the precise route of the conversion of pyruvate to itaconate is still uncertain.

In 1995, Bonnarme et al. [110] used the analytical techniques that combine isotopic tracing, nuclear magnetic resonance spectroscopy, and mass spectroscopy to compare the enzyme systems of intact cells of high- and low-producing strains of *A. terreus*. Results show that itaconate formation requires de novo protein synthesis. During acid formation, TCA cycle intermediates increase in both strains. Furthermore, data showed that both the EMP pathway and the TCA cycle are involved in itaconate biosynthesis. Based on the biosynthetic pathway (Fig. 15), one itaconate molecule is produced from one hexose molecule with a theoretical weight yield of 72.2%. The actual yield should be lower due to the loss of carbon to biomass accumulation and cell maintenance.

Similar to citric acid and other organic acid fermentations by mycelial fungi, dissolved oxygen, trace metal elements, low pH environment, nitrogen and carbon sources, and phosphate have profound impact on itaconic acid accumulation. Figure 16 shows typical kinetics of itaconic acid fermentation [111]. Itaconic acid fermentation is an aerobic process; an increase in the oxygen supply results in an increase in acid yields during submerged fermentation. An interruption in the aeration for 5 min leads to a complete cessation of itaconic acid production, which can only slowly restore 24 h after the resumption of aeration. The effect of aeration interruption is due to cessation of protein synthesis and the loss of ATP supply. Continuous generation of ATP is believed to be required for the transport of produced itaconic acid outside of the cell membrane [112]. The effect of dissolved oxygen concentration and impeller speed on itaconic acid production by *A. terreus* was reported by Park et al. [113].

The production rate and yield of itaconate are influenced by the presence of divalent cations (Fe^{2+}, Zn^{2+}, and Ca^{2+}), physical forms of fungal growth, aeration, and the presence of neutralization agents (e.g. $CaCO_3$). Using *A. terreus* NRRL 1960 as the biocatalyst, Gyamerah [114] indicated that the frayed and

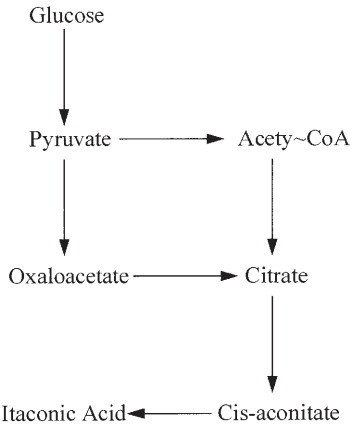

Fig. 15. Suggested pathway of itaconic acid biosynthesis from glucose in *A. terreus* [110]

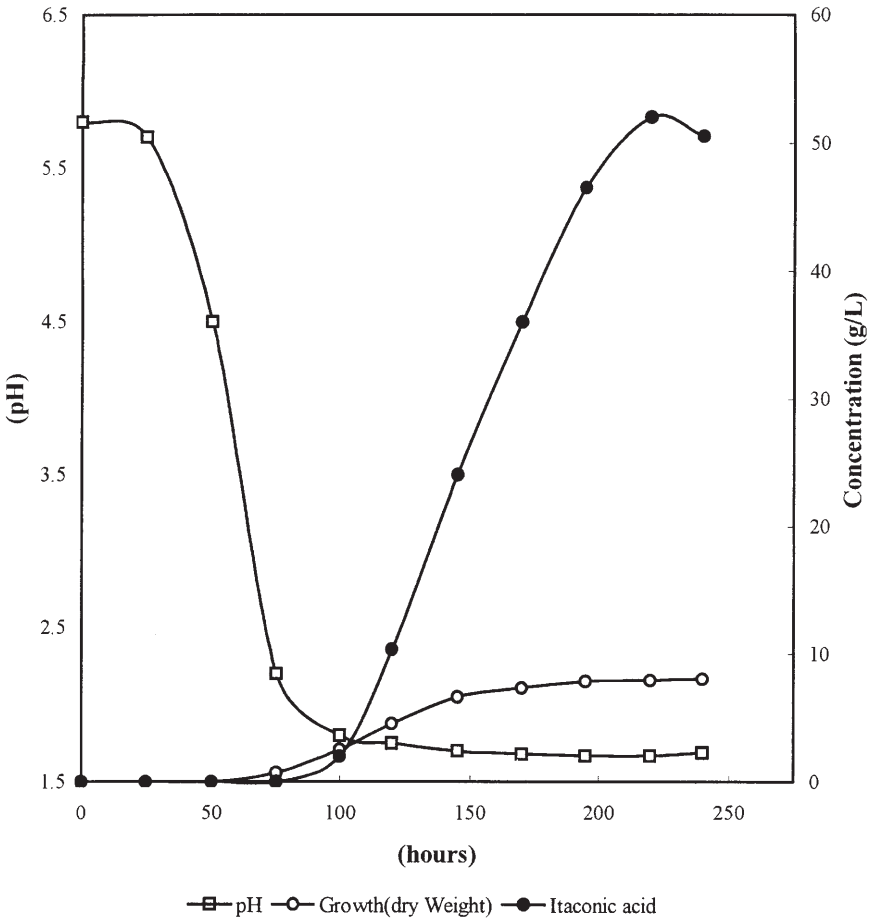

Fig. 16. Kinetics of itaconic production from glucose by *A. terreus* [111]

loose growth form with a size of 0.1 to 0.5 mm in diameter gave the best itaconate yield with the optimal divalent cation concentration shown as follows: Fe^{2+}, 0.06 mg/l; Zn^{2+}, 0.1 mg/l; and Ca^{2+}, 0.23 g/l. The best results showed an itaconic yield of 0.6 g/g sucrose utilized.

Yahiro et al. [115] conducted itaconic acid production from glucose using stirred-tank and air-lift reactors. Results indicate that the air-lift reactor has a much higher productivity (0.64 g/l/h) than the stirred-tank reactor (0.48 g/l/h). Final itaconic acid concentration reached 65 g/l after 96 h of fermentation. Likewise, Okabe et al. [116] used an air-lift bioreactor using a modified draft tube for itaconic acid production and obtained an enhanced itaconic acid yield.

With the objective of producing itaconic acid directly from starchy materials, Kirimura et al. [117] studied the interspecific hydridization between *A. terreus* and *A. usamii* through protoplast fusion. Although *A. terreus* has amylase activity, the activity is not strong enough to produce itaconic acid from starch di-

rectly. On the other hand, *A. usamii*, an *A. niger* related species, shows high productivity of glucoamylase which retains high activity under conditions of low pH (2–4) during fermentation. The resulting stable fusant was able to ferment soluble starch to accumulate 36 g/l itaconate from 120 g/l soluble starch after 6 d incubation. This is, perhaps, the first report of the use of an interspecific protoplast fusion between two different species of *Aspergillus* for the production of itaconic acid from soluble starch.

Itaconic acid production using immobilized cells has also been studied (see [54] for pertinent reference list). The highest productivity of 0.33 g itaconic acid/g of carrier/d was obtained on glucose in a packed-bed reactor which was operated continuously for 4.5 months [118].

9 Conclusions

The organic acids discussed in this chapter are either those manufactured in large volumes or offer vast potential for future development. The production of these acids by fermentation represents a potential route to the production of commodity chemicals from renewable feedstock. The biosyntheses of many of the organic acids by mycelial fungi discussed in this chapter utilize simple sugars through the reductive reactions of the TCA cycle that involves incorporation of carbon dioxide and, theoretically, can result in a net increase in mass relative to the substrate. "Multifunctional" reactive organic acids can be utilized as the feedstock for "biodegradable" polymers or "green" fine chemicals. Many current technologies for the biological conversion of renewable biomass are still in the experimental stage. With the advance of biotechnology through the understanding of biological and biochemical regulations, better biocatalysts can be developed to overcome some of the shortcomings of the existing ones. By experimentation, better bioreactors can be designed and used for the enhancement of productivity and, through better design, more efficient product recovery systems can be obtained. Through relentless research, agricultural residues, food wastes, and municipal wastes can be efficiently converted into valuable products. Overall, the production of organic acids from renewable biomass offers a tremendous opportunity to recycle and reuse natural resources.

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